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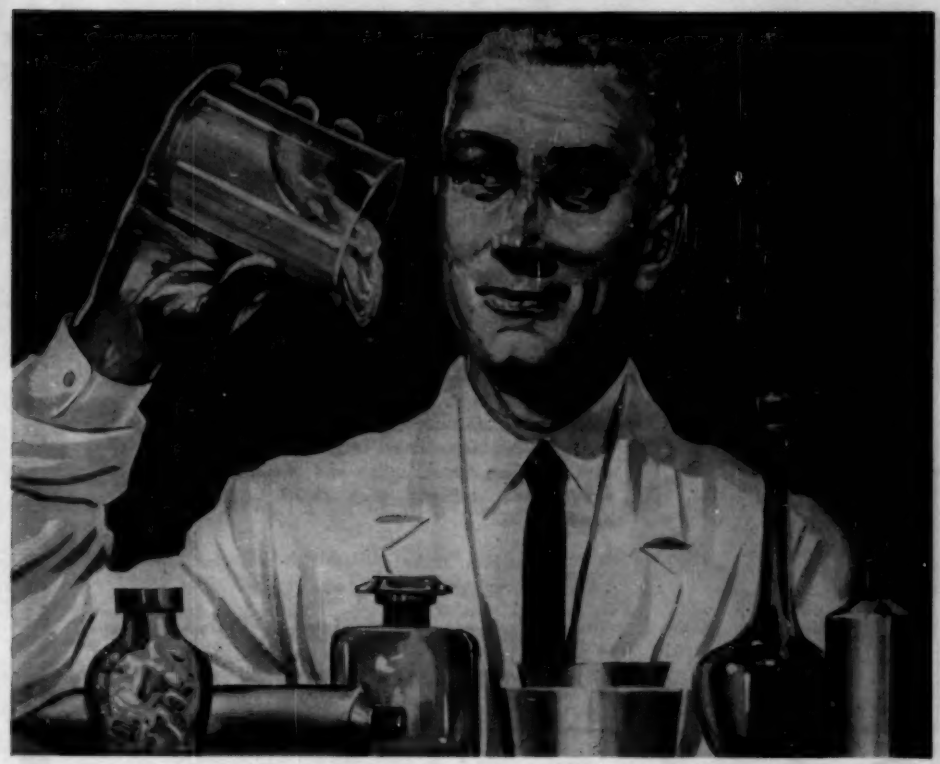

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JOURNAL OF THE AMERICAN PHARMACEUTICAL ASSOCIATION

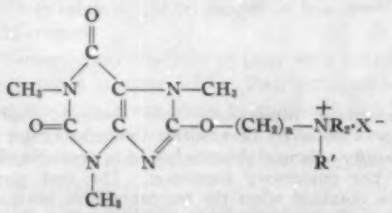
VOLUME XLVII *APRIL 1958* NUMBER 4

Quaternary Salts of 8-(Dialkylaminoalkoxy)-caffeine*

By DIPTISH C. CHAKRAVARTY and JAMES W. JONES†

Three 8-(dialkylaminoalkoxy)-caffeines were prepared by reacting the appropriate sodium dialkylamino alcoholate with 8-chlorocaffeine. Nine quaternary salts were prepared from the above three compounds by reacting each with methyl iodide, ethyl iodide, and benzyl chloride. The melting points and nitrogen content of each compound was determined. The quaternary compounds are to be evaluated for therapeutic activity along with quaternary compounds now being synthesized.

THE SYNTHESIS of a curare-like agent by Bovet (1), using the supposed structure of *d*-tubocurarine as the guide, prompted the investigation of a large number of compounds for their curare-like activity. Among these were the phenylcholine ethers one of which, tri-(β-triethylammoniumethoxy)-benzene-1,2,3-triiodide (Flaxedil), has been used in the clinic (2). The purpose of this work was to prepare some compounds somewhat related to phenylcholine ethers for later testing for possible curare-like activity. Accordingly, some aminoalkylethers, and their quaternary ammonium salts were prepared from 8-chlorocaffeine, having the general formula



where R = CH_3 or C_2H_5 and R'X = CH_3I, C_2H_5I or $C_6H_5CH_2Cl$ and $n = 2$ or 3.

* Received July 12, 1957, from the College of Pharmacy, State University of Iowa, Iowa City.

An abstract of a dissertation submitted by Diptish C. Chakravarty to the Graduate College, State University of Iowa, in partial fulfillment of the requirements for the degree of Master of Science in Pharmacy.

† Professor of Pharmacy, State University of Iowa, Iowa City.

EXPERIMENTAL

8-Chlorocaffeine.—The method of Fischer and Reese (3) was used to prepare 8-chlorocaffeine. Fifty grams of caffeine, dissolved in 400 ml. of dry chloroform, was refluxed over a water bath while a stream of dried chlorine bubbled through it. The white precipitate which first formed disappeared, indicating the completion of the reaction. The mass obtained by evaporation of chloroform was collected on a Büchner funnel, washed with water, and dried.

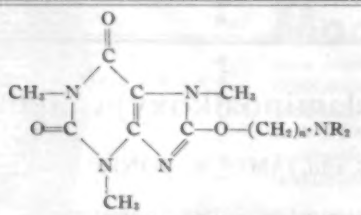
8-(Dialkylaminoalkoxy)-caffeine.—A 2.6-Gm. (0.12 mole) portion of sodium metal, cut into small pieces, was reacted with 0.18 mole of the appropriate alcohol (β-dimethylaminoethanol, β-diethylaminoethanol, or γ-diethylaminopropanol) in 50 ml. of dry benzene. The mixture was warmed over a water bath to complete the reaction. To the benzene solution was added 0.12 mole of 8-chlorocaffeine and the mixture was refluxed until the reaction was completed. The solution was filtered and the filtrate washed with 350-ml. portions of water. The washed solution was dried over anhydrous sodium sulfate and filtered. The benzene was distilled off under reduced pressure and the residue dried. The melting points and analyses are shown in Table I.

Quaternary Salts of 8-(Dialkylaminoalkoxy)-caffeine. Methiodides.—The alkylamino ether (0.10 mole), 50 ml. of absolute ethanol and methyl iodide (0.15 mole) were placed in a Parr bomb and heated at 100° for twelve hours. After cooling, 100 ml. of absolute ethanol was added. The solution was then warmed slightly and filtered. Ether was added to the cooled filtrate to precipitate the quaternary salt, which was filtered out and purified by redissolving

it in absolute ethanol, precipitating with ether and drying.

Ethiodides.—The alkylamino ether (0.10 mole), dissolved in 50 ml. of absolute ethanol, and ethyl iodide (0.20 mole) were placed in a Parr bomb and heated in an oven at 100° for twenty-four hours. After cooling to room temperature, 100 ml. of absolute ethanol was added and the resultant solution was filtered. An excess of ether was added to precipitate the quaternary salt. The mixture was kept cold overnight, after which the salt was filtered out and dried.

TABLE I.—8-(DIALKYLAMINOALKOXY)-CAFFEINE



R	n	M. P., °C.	Yield, %	N, % Calcd.	N, % Found
CH_3	2	169	36	24.9	24.3
CH_3CH_2	2	138	38.6	22.6	22.3
CH_3CH_2	3	165–167	23	21.6	21.3

The melting points, analyses and yields of the quaternary salts are given in Table II.

DISCUSSION

The methods employed to obtain the alkylamino ethers and their respective quaternary salts had to be modified slightly as the length of the alkyl chain attached to the oxygen increased and as the size of the groups attached to the nitrogen increased.

The sodium alcoholate of dimethylaminoethanol was obtained at room temperature while for diethylaminoethanol it was necessary to warm the mixture. To obtain the sodium alcoholate from diethylaminopropanol it was necessary to reflux on a water bath for two hours.

Similarly, 8-chlorocaffeine reacted with sodium diethylaminopropylate very slowly and refluxing with constant stirring for four hours was required to complete the reaction.

In the synthesis of the quaternary salt, methyl iodide and 8-(dimethylaminoethoxy)-caffeine had to be refluxed for twelve hours to obtain only a fair yield. The Parr bomb method increased the yield appreciably. 8-(Diethylaminoethoxy)-caffeine did not produce any appreciable yield of quaternary salt with ethyl iodide even after refluxing for seventy-two hours. The Parr bomb method was again found to be the only one which produced a workable quantity of the quaternary compound.

Much difficulty was encountered in preparing the quaternary compounds with benzyl chloride It was observed that neither temperature nor the quantity of benzyl chloride had an appreciable effect on the quaternary formation. The best results were obtained when the reactants were heated at 80° in a sealed container for ninety-six hours.

TABLE II.—QUATERNARY SALTS OF 8-(DIALKYLAMINOALKOXY)-CAFFEINE

R	n	R′	X	Yield, %	M. P., °C	N, % Calcd.	N, % Found
CH_3	2	Me	I	21	109–112[a]	16.5	16.3
		Et	I	20	204–206[a]	16.02	16.0
		Benz	Cl	15	142–145	17.1	17.7
CH_3CH_2	2	Me	I	20	193–195[a]	15.5	15.2
		Et	I	18	185–187[a]	15.05	15.0
		Benz	Cl	20	170–172	16.07	15.99
CH_3CH_2	3	Me	I	15	168–172[a]	15.05	14.98
		Et	I	20	188–190[a]	14.6	14.3
		Benz	Cl	35	180–183	15.1	15.0

[a] With decomposition.

Benzchlorides.—The Parr bomb did not give good results, so the following method was used. A strong-walled 1-L. bottle fitted with rubber stopper was used. The alkylamino ether (0.10 mole) was dissolved in 50 ml. of absolute ethanol and benzyl chloride (0.30 mole) was added to the solution. The rubber stopper was secured with a wire and the bottle was placed in a tin container and kept in an oven at 80° for ninety-six hours. The mixture was then diluted with 100 ml. of absolute ethanol, stirred well and filtered. An excess of ether was added to precipitate the quaternary salts. The salt was filtered out, washed with more ether and dried.

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(2) Bovet, D., Depierre, F., and de Lestrange, Y., *Compt. rend.*, **233**, 74(1947).
(3) Fischer, E., and Reese, L., *Ann.*, **221**, 336(1883).

Conditions for the Preservation of Gum Tragacanth Jellies*

By ABRAHAM TAUB, WILLIAM A. MEER,† and LUCY W. CLAUSEN

The implication that the effectiveness of certain preservatives for tragacanth jelly is reduced by either adsorption or chemical interaction between the gum and the preservative is subjected to further experimentation in this study. Results indicate that for the organisms selected in this study, effective preservation may be attained if adequate concentration of preservative and optimum pH range is selected. The organisms selected were *Micrococcus pyogenes* var. *aureus, Bacillus subtilis, Escherichia coli,* and *Candida albicans.* The preservatives studied were a combination of methyl and propyl parabens, cholorobutanol, and benzoic acid. The optimum pH range was found to be below five, but under certain conditions even seven proved to be satisfactory.

In a recently reported study (1) it is concluded that in gum tragacanth jellies buffered at pH 7, the bactericidal activities of some commonly used preservative agents are neutralized by the gum either at the time of manufacture or upon storage.

Since pharmaceutical jellies containing gum tragacanth have been marketed for many years with apparent satisfactory preservation, it was the object of this study to determine under what conditions such preservation could be attained, and also to examine further the possible inactivation of the preservative agent by the gum. The study was limited to a selection of three preservative agents in jellies buffered at different pH's; activity was tested against three bacterial organisms and one fungus.

Gum tragacanth has been widely investigated with respect to its physical and chemical properties. A recent review (2) comprehensively covers the subject.

Powdered ribbon tragacanth, U. S. P., type E 1 (Meer Corp., N. Y. C.) was used in the preparation of all jellies. The gum is a fine, white, freely flowing powder, 100% of which passes through a 150-mesh screen. It originated in Iran from the 1956 crop.

Various concentrations of gum were tested in preparing a suitable jelly. Two per cent w/w gum tragacanth was found to produce a satisfactory jelly of good consistency. The jellies prepared neither flowed too freely nor were they extremely viscous.

The jellies were prepared using 5% w/w propylene glycol as a dispersing agent for the gum. This concentration was found not to exhibit any inhibitory effect on bacterial and mold growth.

The use of McIlvaine's buffer in a threefold dilution with distilled water enabled jellies ranging from pH 3 to pH 7 to be prepared.

A combination of the methyl (0.2%) and propyl (0.05%) esters of para-hydroxybenzoic acid, benzoic acid 0.2% alone, and chlorobutanol 0.5% alone, were used in the preservation of the prepared jellies. These agents were chosen due to their widespread usage and effectiveness, their general compatibility, and their nontoxicity (2–5).

The role of pH in preservation was also considered. Benzoic acid is an excellent preservative in media which are strongly acidic. It is, however, much less effective in weakly acidic or neutral compositions. The optimum pH range for chlorobutanol is also on the acid side, pH 3–6. The parabens are active in acid, neutral, and alkaline media (6).

Moreover, for any given bacterial culture there exists a pH range permitting growth, beyond which the hydrogen or hydroxyl ion concentration exhibits bactericidal action. Thus pH alone may impart preservative qualities.

Many bacteria do not grow at pH 5, and with very few exceptions, bacteria do not survive and grow at pH 3. This is an important consideration, for a jelly may thus be preserved for a greater length of time by the use of low pH in conjunction with a preservative (7).

It may, therefore, be unrealistic to base a study solely on jellies at pH 7, especially since many tragacanth jellies containing therapeutic agents now in use are on the acid side.

In order to determine the efficacy of preservative action, test organisms were added to jellies and controls in known quantities, and samples were taken at predetermined time intervals to determine the effects of the preservative.

* Received May 1, 1957, from the College of Pharmacy, Columbia University, New York, N. Y.
Presented to the Scientific Section, A. Ph. A., New York Meeting, April 1957.
Abstracted from a thesis submitted by William A. Meer in partial fulfillment of the requirements for the degree of Master of Science.
† Present Address: School of Pharmacy, University of Connecticut, Storrs, Conn.

To obtain evidence of preservation, representative contaminating organisms were chosen. Organisms naturally present in the gum or at the site of use of tragacanth jellies, in addition to those demonstrating different bacterial characteristics were utilized.

Micrococcus pyogenes var. *aureus*, *Bacillus subtilis*, *Escherichia coli*, and *Candida albicans* were selected as covering a fairly wide range of usage.

EXPERIMENTAL

The following general formula and method of preparation was employed for the jellies. Two per cent w/w of gum was wetted with 5% w/w propylene glycol and thoroughly mixed. The diluted buffer solution containing the total amount of water and the selected preservative, when used, was then added at one time with constant stirring until a gel formed. During the entire procedure, as far as possible, aseptic conditions were maintained.

The finished jellies were translucent and free from lump formation, and the incorporation of air bubbles was at a minimum. Uniform viscosities were obtained and the jellies were of good ointment-like consistency.

Viscosity was also used as a criterion for standardizing the finished product. Viscosity measurements were taken using a Brookfield Model LVF viscosimeter with Helipath stand and spindle attachments. Jellies having a viscosity of 45,000 centipoises were used in testing.

The testing procedure consisted of inoculating previously sterilized jellies and sterile aqueous control solutions with a standard number (approximately 100,000) of organisms per 10 Gm. of jelly or 10 ml. of solution. Sterilization of the jellies was found to be necessary after preliminary testing gave erratic results showing overgrowth and contamination.

Determinations of the efficacy of the various preservatives were then carried out by withdrawing samples at time intervals ranging from ten minutes to one week and inoculating in culture media. After plating, direct colony counts were taken as indications of the number of organisms present in the samples tested.

In transfer and plating operation, suitable dilutions were carried out to insure that no bacteriostatic concentrations of preservative would be carried over into the plating medium. This was verified by the results obtained.

Transfers were made in nutrient broth, and plating was accomplished by the use of a layering technique employing soft agar poured on nutrient agar. This method provided for evenly distributed colonies which remained on the surface of the agar rather than being diffused throughout, enabling more simplified and accurate counts to be made.

The nutrient broth,[1] soft agar, and nutrient agar[1] used were found to be suitable for the growth of all the test organisms. The media utilized for transfer and plating was at pH 7.

The jellies were tested, buffered over a pH range of 3 to 7. This range was selected since many commercially prepared jellies employing tragacanth are on the acid side.

For control purposes the effect of pH on inoculating organisms in unpreserved jellies and in aqueous solutions was also determined. In order to determine whether a reduction in bacteriostatic activity took place in the presence of gum tragacanth, aqueous solutions having the same formulations but omitting the gum were tested.

In Table I is listed the influence of pH on colony

[1] Difco formulas.

TABLE I.—THE INFLUENCE OF pH ON UNPRESERVED, STERILE JELLIES CONTAINING 2% GUM TRAGACANTH, AND ON CONTROL AQUEOUS SOLUTIONS

Organism	pH	Colony Count after 10 Min. Jelly	10 Min. Sol.	½ Hour Jelly	½ Hour Sol.	1 Hour Jelly	1 Hour Sol.	1 Day Jelly	1 Day Sol.	2 Days Jelly	2 Days Sol.	1 Week Jelly	1 Week Sol.
Micrococcus pyogenes var. *aureus* 209	3	231	0	223	0	96	0	0	0	0	0	0	0
	4	267	86	190	2	189	0	34	0	27	0	0	0
	5	345	156	321	108	204	52	81	0	57	0	0	0
	6	374	178	323	102	267	88	356	49	1290	5	[b]	0
	7	394	235	379	166	306	115	559	59	4320	17	[b]	0
Bacillus subtilis 9524	3	0	0	0	0	0	0	0	0	0	0	0	0
	4	358	0	70	0	33	0	0	0	0	0	0	0
	5	381	348	256	361	426	540	695	127	827	1	1153	0
	6	446	388	561	390	586	676	693	133	952	10	2131	0
	7	385	370	436	385	636	520	712	740	801	1421	1609	[b]
Escherichia coli 9637	3	115	8	23	0	4	0	0	0	0	0	0	0
	4	128	47	121	19	120	14	65	0	18	0	4	0
	5	126	85	135	70	191	100	[a]	4	[a]	3	[a]	0
	6	137	109	130	66	140	53	[a]	[a]	[a]	[a]	[a]	[a]
	7	120	127	135	138	140	174	[a]	[a]	[a]	[a]	[a]	[a]
Candida albicans 10259	3	320	245	140	152	207	155	2730	385	5780	1841	[b]	[b]
	4	472	255	240	235	267	326	2380	569	6441	3014	[b]	[b]
	5	440	356	160	315	346	318	4094	369	6238	2608	[b]	[b]
	6	395	229	242	331	456	449	4261	636	5699	2385	[b]	[b]
	7	338	329	256	243	508	278	3244	421	5292	2033	[b]	[b]

[a] Too many organisms per plate for an accurate count to be taken: over 20,000.
[b] No counts taken since counts at previous time period were very high.

counts of 2% tragacanth jellies, and of control aqueous solutions, which contain no added preservatives and which have been inoculated after sterilization with selected organisms.

In all the following tables the colony counts represent the number of organisms isolated from 0.1 Gm. of jelly or 0.1 ml. of solution.

After examining the effect of pH on the test organisms, similar jellies and solutions, with preservatives added, were studied. Where pH alone was sufficient to inhibit growth, no experiments were set up with added preservatives at these pH's. The results obtained appear in Table II.

The results in Table II indicate that the presence of gum tragacanth reduces to some extent the activity of the preservatives tested. This retardant action of the gum is more noticeable at higher pH's. It is evidenced by longer periods of time necessary for inhibition of growth, and by higher colony counts than those obtained in the control aqueous solution; such reduction, however, is not sufficiently great to interfere with their usefulness as preservatives of tragacanth jellies.

The observation that growth at times occurs after an initial period of partial reduction also indicates that the inactivation of the preservative is a process which may be influenced by aging. Thus experiments were set up so that preserved jellies and solutions might be tested after storage for 28 days. At the end of this period the jellies were inoculated with the specific organisms and tested similarly to the unstored jellies. The results are summarized in Table III.

The data presented thus far are in reference to sterilized jellies. Since most jellies are not usually sterilized prior to the addition of preservatives, a series was made up to determine whether bacteriostatic agents are effective in nonsterile jellies. The results obtained are shown in Table IV.

DISCUSSION AND CONCLUSIONS

The results of this study show that gum tragacanth jellies can be effectively preserved.

Benzoic acid was found to be ineffective as a

TABLE II.—THE BACTERIOSTATIC ACTIVITY OF FRESHLY PREPARED, PRESERVED, STERILE JELLIES CONTAINING 2% GUM TRAGACANTH, AND OF CONTROL PRESERVED AQUEOUS SOLUTIONS

			Colony Count after											
			10 Min.		½ Hour		1 Hour		1 Day		2 Days		1 Week	
Preservative	Organism	pH	Jelly	Sol.	Jelly	Sol.	Jelly	Sol.	Jelly	Sol.	Jelly	Sol.	Jelly	Sol.
Benzoic Acid 0.2%	*Micrococcus Aureus* 209	5	208	60	204	0	127	0	64	0	0	0	0	0
		7	320	312	267	221	153	157	305	0	330	0	1275	0
	Bacillus subtilis 9524	5	153	134	135	95	86	9	78	3	6	2	39	2
		7	138	127	129	110	92	19	90	11	4	12	49	8
	Escherichia coli 9637	4	129	69	96	5	9	0	0	0	0	0	0	0
		5	214	116	185	86	111	73	21	0	0	0	0	0
		7	163	124	112	102	74	100	238	34	211	83	234	196
	Candida Albicans 10259	3	0	0	0	0	0	0	0	0	0	0	0	0
		5	141	7	20	0	7	0	0	0	0	0	0	0
		7	161	133	85	64	41	52	23	22	20	17	3887	0
Chlorobutanol 0.5%	*Micrococcus Aureus* 209	5	203	0	204	0	33	0	16	0	0	0	0	0
		7	332	30	326	0	254	0	114	0	60	0	0	0
	Bacillus subtilis 9524	5	167	114	50	41	42	8	1	4	2	2	30	3
		7	186	186	116	108	47	22	9	4	3	5	30	3
	Escherichia coli 9637	4	229	0	157	0	66	0	0	0	0	0	0	0
		5	259	5	125	0	107	0	93	0	17	0	0	0
		7	233	213	202	192	214	165	146	101	51	7	27	0
	Candida albicans 10259	3	0	0	0	0	0	0	0	0	0	0	0	0
		5	13	0	0	0	0	0	0	0	0	0	0	0
		7	29	0	25	0	20	0	21	0	20	0	4922	0
Methyl 0.2% and Propyl 0.05% Parabens	*Micrococcus aureus* 209	5	0	0	0	0	0	0	0	0	0	0	0	0
		7	76	10	12	0	0	0	0	0	0	0	0	0
	Bacillus subtilis 9524	5	143	152	2	2	2	2	1	1	3	2	30	9
		7	155	124	2	9	3	6	1	4	1	2	35	4
	Escherichia coli 9637	4	0	0	0	0	0	0	0	0	0	0	0	0
		5	0	0	0	0	0	0	0	0	0	0	0	0
		7	0	0	0	0	0	0	0	0	0	0	0	0
	Candida albicans 10259	3	0	0	0	0	0	0	0	0	0	0	0	0
		5	0	0	0	0	0	0	0	0	0	0	0	0
		7	0	0	0	0	0	0	0	0	0	0	0	0

TABLE III.—THE BACTERIOSTATIC ACTIVITY OF PRESERVED, STERILE JELLIES CONTAINING 2% GUM TRAGACANTH, AND OF CONTROL AQUEOUS SOLUTIONS, AFTER TWENTY-EIGHT DAY STORAGE PERIOD

			Colony Count after											
			10 Min.		½ Hour		1 Hour		1 Day		2 Days		1 Week	
Preservative	Organism	pH	Jelly	Sol.	Jelly	Sol.	Jelly	Sol.	Jelly	Sol.	Jelly	Sol.	Jelly	Sol.
Benzoic Acid 0.2%	*Micrococcus aureus* 209	5	218	135	210	83	136	5	71	0	0	0	0	0
		7	313	335	287	227	216	121	293	113	350	22	1780	0
	Bacillus subtilis 9524	5	150	141	141	102	72	2	22	1	5	1	28	1
		7	129	132	132	121	94	27	75	4	9	7	40	5
	Escherichia coli 9637	4	135	85	62	19	8	0	0	0	0	0	0	0
		5	190	159	121	140	87	118	11	0	0	0	0	0
		7	160	116	108	106	83	75	142	59	171	58	257	128
	Candida albicans 10259	3	0	0	0	0	0	0	0	0	0	0	0	0
		5	150	122	30	97	11	0	0	0	0	0	0	0
		7	155	127	100	99	52	777	23	62	20	111	1878	204
Chlorobutanol 0.5%	*Micrococcus aureus* 209	5	235	0	250	0	180	0	31	0	0	0	0	0
		7	329	86	318	0	324	0	254	0	134	0	120	0
	Bacillus subtilis 9524	5	137	124	132	34	28	0	2	2	1	1	24	5
		7	128	122	118	93	57	19	8	2	2	1	18	6
	Escherichia coli 9637	4	160	1	145	0	46	0	0	0	0	0	0	0
		5	159	20	111	0	105	0	88	0	11	0	0	0
		7	183	220	174	212	199	118	140	96	73	0	23	0
	Candida albicans 10259	3	160	97	122	91	134	96	171	0	172	0	1306	0
		5	175	135	126	127	104	82	100	12	845	3	6360	0
		7	159	137	162	125	151	172	163	149	941	126	9953	197
Methyl 0.2% and Propyl 0.05% Parabens	*Micrococcus aureus* 209	5	0	0	0	0	0	0	0	0	0	0	0	0
		7	81	18	20	0	0	0	0	0	0	0	0	0
	Bacillus subtilis 9524	5	131	121	3	2	2	0	5	1	6	3	23	6
		7	147	126	0	3	3	1	1	2	8	1	32	3
	Escherichia coli 9637	4	0	0	0	0	0	0	0	0	0	0	0	0
		5	0	0	0	0	0	0	0	0	0	0	0	0
		7	0	0	0	0	0	0	0	0	0	0	0	0
	Candida albicans 10259	3	0	0	0	0	0	0	0	0	0	0	0	0
		5	0	0	0	0	0	0	0	0	0	0	0	0
		7	0	0	0	0	0	0	0	0	0	0	0	0

TABLE IV.—THE BACTERIOSTATIC ACTIVITY OF UNPRESERVED AND PRESERVED, NONSTERILE JELLIES CONTAINING 2% GUM TRAGACANTH

		Colony Count[a] after			
Preservative	pH	1 Hour	1 Day	2 Days	28 Days
Benzoic Acid 0.2%	3	13	5	6	0
	4	18	6	5	1
	5	15	4	7	9
	7	571	596	583	Mold growth
Chlorobutanol 0.5%	3	36	7	8	0
	4	24	4	6	1
	5	22	7	5	2
	7	25	9	7	4
Methyl 0.2% and Propyl 0.05% Parabens	3	12	2	3	0
	4	16	3	4	2
	5	14	9	3	2
	7	17	4	5	3
Unpreserved jellies	3	Visible mold growth after 2 weeks			
	4				
	5				
	6	Liquefaction after 2 weeks			
	7				

[a] Organism representing naturally occurring contaminants.

preservative at pH 7 for the organisms tested, but at pH 5 it demonstrated good qualities. This was to be expected as benzoic acid at pH 7 is highly ionized losing much of its activity.

Chlorobutanol proved to exert a measurable preservative action at pH 5 against all four organisms. At pH 7 it was effective in inhibiting three of the organisms, but in the case of *Candida albicans* growth was manifested at pH 7. After 28 days storage this preservative was similarly effective against *Micrococcus aureus*, *Bacillus subtilis*, and *Escherichia coli*. A marked decrease in bacteriostatic activity at pH's 3, 5, and 7 against *Candida albicans*, however, was noted. This inactivation was much greater in the presence of gum tragacanth than in the aqueous control solutions. The breakdown of effectiveness at all pH's, particularly at pH 7, however, was most likely due to the instability of chlorobutanol especially after being subjected to autoclaving temperatures and storage.

The influence of the gum on the bacteriostatic activity of chlorobutanol, which was not noticeable against the organisms other than *Candida albicans*, may have been due to a reduction of the concentration of this preservative to below an effective level. Enough loss of chlorobutanol may have resulted upon aging to reduce the amount of preservative to a point below bacteriostatic effectiveness against this particular organism.

Gum tragacanth exerted no marked deleterious effect on the bacteriostatic activity of the parabens. It appears probable that an effective concentration of the parabens still remained in the jelly even after any possible slight adsorption by the gum.

As evidenced by the tests on the nonsterilized jellies, containing only the naturally occurring organisms, effective bacteriostasis may still be maintained even after aging, in the cases of chlorobutanol and the parabens, and to a limited extent in the case of benzoic acid.

The preserved sterile jellies after inoculation exhibited some killing power, but the preservative agents were mainly effective in keeping the number of organisms to a minimum. This was most noticeable in the case of *Bacillus subtilis* where complete destruction was never recorded.

Bactericidal activity is not requisite for an agent used for the preservation of a jelly; a jelly may be well preserved by a bacteriostatic agent in adequate concentration.

The fact that preservation was effective after a short period of time in nonsterile jellies, 1 to 2 days, at pH 5 is also an important consideration, for industrial practices do not usually call for sterilization of jellies. Complete inhibition at pH 3 also demonstrates that self sterilization may be possible.

Where jellies are to be used shortly after preparation, sterilization may be necessary, as the time of contact may be insufficient to effectively control any contaminating organisms. In most instances, however, a storage period of several hours to two days would be within the scope of standard manufacturing practices and under such conditions jellies could be preserved by the use of bacteriostatic agents alone without the need of autoclaving techniques.

The effect of pH is important in preparing jellies. Of the preservatives employed, benzoic acid proved ineffective, and chlorobutanol only partially effective at neutral pH. Since most pharmaceutical products having a tragacanth jelly base are prepared at pH's below 7, the selection of pH 7 for evaluating the preservative action of bacteriostatic agents incorporated in such jellies is unduly limited and may result in conclusions which may not truly reflect the usefulness of this widely applicable gum.

The combination of methyl and propyl parabens was much more effective than either chlorobutanol or benzoic acid. The parabens possessed good antibacterial and antifungal activity at pH 7 and below.

To the extent that the specific organisms used in this study as well as the organisms commonly present in this natural gum are indicative of the factors causing spoilage of tragacanth jellies, it is possible to preserve the jellies against such decomposition by suitable selection of pH buffer and adequate concentration of preservative. Benzoic acid, at pH 5 or below, at a concentration of 0.2% is effective but to a lesser degree than the parabens. Chlorobutanol is only partly effective at a concentration of 0.5% at pH 5 or below. After storage, however, it is not effective against *Candida albicans*. Optimum conditions were obtained with a combination of methyl and propyl parabens at a concentration of 0.2% and 0.05% respectively, at a pH of 7 or below.

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Some Anticonvulsant, Skeletal Muscle Relaxing, and Toxic Properties of a Series of Substituted Cyclohexanones*

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A series of substituted cyclohexanones was screened for anticonvulsant, skeletal muscle relaxing and toxic properties in mice, and for those compounds that were selected for further study, ED_{50}, PD_{50}, and LD_{50} values were determined. Although the series in general, possesses low water solubility, individual members are rapidly absorbed following intraperitoneal injection of suspensions of the compounds. 2-(*o*-Methylphenyl)cyclohexanone was one of the most active compounds in the series, both as an anticonvulsant and as a muscle relaxant. Because its action resembled that of mephenesin, a comparison of the two compounds was made. The effect of the ortho-isomer on strychnine convulsions was also studied.

PREVIOUS INVESTIGATIONS on α-phenylcarbonyl compounds (1–3) suggested the desirability of studying some new α-substituted cyclohexanones (4). Accordingly, a series of 2-phenylcyclohexanone derivatives was prepared (4), several members of which showed a rather high degree of skeletal muscle paralyzing and anticonvulsant action. Since most of the compounds are new, the anticonvulsant, muscle relaxant, and acute toxic properties of the series were studied and are being reported at this time.

With the exception of 2-(*o*-chlorophenyl)-cyclohexanone and 2-(*m*-chlorophenyl)-cyclohexanone, the syntheses of all compounds studied in this investigation have been reported in recent papers (5, 6). The method of preparing the *o*- and *m*-chlorophenyl isomers is essentially the same as that reported for the *p*-isomer. Detailed synthesis and physical constants of intermediates will be reported elsewhere.

EXPERIMENTAL

Albino mice of the Webster strain, consisting largely of male animals weighing 18–22 Gm. were used in all anticonvulsant, muscle relaxant, and toxicity experiments. Compounds were prepared in either suspension (for solids) or emulsion (for liquids) form in concentrations of 50 mg./ml. of 6% acacia solution, and administered intraperitoneally unless otherwise specified. Mice used in the strychnine and semichronic toxicity experiments were fasted twelve hours prior to use, whereas in the anticonvulsant and muscle relaxant experiments they had access to both food and water.

ED_{50}, PD_{50}, and LD_{50} values with 95% confidence limits were determined for the anticonvulsant, muscle relaxing, and acute toxic properties, respectively, by the method of Litchfield and Wilcoxon (7). In each experiment at least three dosage points were established between the limits of 100% and 0% effect.

Anticonvulsant.—Anticonvulsant properties were studied by the maximal electroshock seizure method (MES) of Swinyard, *et al.* (8). By this method the property of a compound to abolish the hind leg extensor thrust of the maximal seizure induced by 50 mA. of current applied through corneal electrodes for 0.2 second is studied. For screening purposes, three or more mice were injected with a dose of 1,000 mg./Kg. and shocked after 15, 30, 60, and 120 minutes. Compounds that gave no protection against the maximal seizure at the above dose level were considered "inactive." Compounds that gave 100% protection at 1,000 mg./Kg. dose were administered at decreasing dose levels, each one-half that of the preceding dose. One compound, 2-(*o*-methylphenyl)cyclohexanone, was studied for its anticonvulsant property against convulsions induced by pentylenetetrazol, according to the method of Goodman, *et al.* (9). Seizures were elicited by the rapid intravenous injection of pentylenetetrazol (38 mg./Kg., in a 0.5% solution). Compounds that showed anticonvulsant activity in 1,000 mg./Kg. dose or lower during screening experiments were studied in greater detail for anticonvulsant, muscle relaxant and toxicity properties.

Muscle Relaxant.—The series of compounds was screened for muscle relaxant properties simultaneously with the screening for anticonvulsant properties. Animals were considered paralyzed when they had lost the righting reflex for one minute. Those compounds that were investigated beyond the screening stage for anticonvulsant properties were also studied further for their muscle relaxant properties.

Toxicity.—A semichronic toxicity experiment was conducted over a six-week period in which an experimental group of both male and female mice

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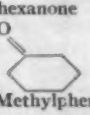
received intraperitoneally 2-(*o*-methylphenyl)cyclohexanone in doses of 180 mg./Kg. twice weekly. Controls received acacia solution. Serial sacrifices were made at two-week intervals at which time tissues of the vital organs were prepared for microscopic examination.

2-(*o*-Methylphenyl)cyclohexanone vs. Strychnine.—The effect of 2-(*o*-methylphenyl)cyclohexanone on strychnine convulsions was studied in three groups of twenty mice each. Strychnine sulfate was administered intraperitoneally in a solution containing 18 mg./100 ml. of solution. Controls received strychnine sulfate, 2.5 mg./Kg., whereas the first and second experimental groups received, in addition to strychnine, 350 mg./Kg. (approximate PD_{50} dose), and 800 mg./Kg. of 2-(*o*-methylphenyl)cyclohexanone, respectively. The animals in the first group received the strychnine twenty minutes after the cyclohexanone, whereas in the second group, it was administered after ten minutes.

TABLE I.—THE ANTICONVULSANT AND MUSCLE RELAXING ACTIVITIES OF A SERIES OF SUBSTITUTED CYCLOHEXANONES IN WHITE MICE (SCREENING EXPERIMENT)

Compound	Anticonvulsant Activity,[a] Animals Protected/ Animals Tested		Muscle Relaxing Activity,[b] Animals Paralyzed/ Animals Tested	
Cyclohexanone 	600[c]	4/4	1,000[c]	4/4
	500	3/5	800	0/6
	350	0/5		
2-(*m*-Methylphenyl)-	500	3/3	1,000	6/6
	250	0/3	500	0/6
2-(*o*-Methylphenyl)-	275	6/6	475	6/6
	125	1/6	275	0/6
2-(*p*-Methylphenyl)-	1,000	1/10	1,000[d]	3/10
2-(*m*-Chlorophenyl)-	500	3/3	1,000	2/3
	250	2/3	500	0/3
	125	1/3		
2-(*o*-Chlorophenyl)-	500	3/3	1,000	1/3
	250	3/3	500	0/3
	125	0/3		
2-(*p*-Chlorophenyl)-	500	3/3	1,000	1/3
	250	2/5		
2-(*p*-Bromophenyl)-	1,000	1/3	1,000	0/3
	500	2/3		
	250	0/3		
2-(*p*-Hydroxyphenyl)-	1.000	2/3	1,000	0/3
	500	2/3		
	250	0/3		
2-(*p*-Isopropylphenyl)-	2,000	0/4	2,000	0/4
2-(α-Hydroxy-*p*-bromobenzyl)-	500	3/3	1.000	2/3
2-(α-Hydroxy-*p*-iodobenzyl)-	1,000	0/3	500	0/3
			1,000	0/3
2-(*p*-Bromobenzylidene)-	500	2/3	1,000	0/3
	250	0/2		
2-(*p*-Bromobenzyl)-	500	3/3	500	0/3
	250	0/3		
2,6-bis(*p*-Chlorobenzylidene)-	1,000	0/3	1,000	0/3
2,6-bis(*p*-Chlorobenzyl)-	1,000	0/3	1,000	0/3
6-*p*-Nitrophenyl-3-cyclohexen-1-one	1,000	1/3	1,000	0/3

[a] The anticonvulsant activity was measured by the maximal electroshock seizure test.
[b] Indicated when animals had lost the righting reflex for at least one minute.
[c] Figures in this column are mg./Kg.
[d] Three deaths.

RESULTS

The results of the screening experiments are presented in Table I, whereas those from more refined experiments are presented in Table II.

2-(*o*-Methylphenyl)cyclohexanone Compared with Mephenesin.—Because of the similarity of their paralytic effects, (2-*o*-methylphenyl)cyclohexanone and mephenesin were studied simultaneously in mice. The anticonvulsant property of mephenesin was also determined after intraperitoneal doses of 1/1.92 of the PD_{50} dose, since the PD_{50}:ED_{50} ratio of the cyclohexanone compound was 1.92. At this dose (ED_{50}) 2-(*o*-methylphenyl)cyclohexanone exhibited anticonvulsant action, whereas mephenesin did not (at either fifteen or thirty minutes). Mephenesin, at its PD_{50} dose (200 mg./Kg.) did abolish the hind leg extensor thrust induced by the maximal shock seizure, but about half of the animals had lost the righting reflex prior to shocking.

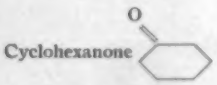
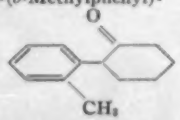
Methylphenylcyclohexanone in Rats.—The three isomeric 2-(methylphenyl)cyclohexanones were studied in rats (Long Evans' strain, 100 Gm.) for both anticonvulsant and paralyzing properties. At 1,000 mg./Kg. dose, *only* the *o*-isomer caused paralysis. Against the maximal electroshock, 4/4 rats were protected at 125 mg./Kg. suggesting a greater anticonvulsant action in rats than in mice.

DISCUSSION

Although cyclohexanone has been a useful chemical intermediate for many years, only a few investigations regarding its biological properties have been made. Treon, *et al.* (10), observed marked and persistent "narcosis" from orally administered cyclohexanone in rabbits, and estimated the "minimum lethal dose ... by oral administration... (to be)...between 1.6 and 1.0 Gm./Kg." Cyclohexanone is rapidly absorbed when administered to animals, as shown by the work of Treon, *et al.* (10), in addition to that of the present study.

The water solubility of the compounds comprising the series reported in this paper, in general, is less than that of cyclohexanone. However, it is interesting to note the rapidity with which many of them are absorbed following intraperitoneal administration. For example, the *o*-, *m*-, and *p*- isomers of 2-(methylphenyl)cyclohexanone produced ataxia and gave evidence of central depression in a matter of minutes. Of these isomers the ortho member was considerably the most active, both as an anticonvulsant and as a paralyzant, and loss of the righting reflex occurred in less than five minutes with doses of 475 mg./Kg. This member was not only rapidly absorbed in mice, but likewise rapidly eliminated or detoxified, since it failed to protect animals against the electroshock convulsion longer than thirty to forty-five minutes and recovery from the paralytic effects occurred in about forty-five minutes following doses approximating those of the ED_{50} and PD_{50}, respectively. Why the ortho compound should be more active than the meta and para isomers is not easily explained. An explanation on the basis of

TABLE II.—THE ANTICONVULSANT AND MUSCLE RELAXING PROPERTIES AND THE ACUTE TOXICITY OF A SERIES OF SUBSTITUTED CYCLOHEXANONES AND REFERENCE COMPOUNDS IN WHITE MICE[a]

Compound	ED_{50}[b] mg./Kg.	ED_{50}[b] mM./Kg.	PD_{50} mg./Kg.	PD_{50} mM./Kg.	LD_{50} mg./Kg.	LD_{50} mM./Kg.	Ratio, LD_{50}/PD_{50}[c]	Ratio, LD_{50}/ED_{50}[c]	Ratio, PD_{50}/ED_{50}[c]
 Cyclohexanone	460 (397–535)	4.68	900 (840–965)	9.17	1,750 (1,580–1,950)	17.8	1.94	3.80	1.96
2-(*o*-Methylphenyl)-	180 (143–227)	0.96	345 (314–380)	1.84	1,200 (1,090–1,320)	6.38	3.48	6.66	1.92
	204 (154–272)	1.08	(Metrazol Test)					5.88	1.69
	730 (570–1,000)	3.88	(Intragastric Administration)				..	..	..	..
2-(*p*-Aminophenyl)-hydrochloride	130 (108–156)	0.58	410 (354–475)	1.82	About 750	~3.4	~1.8	~5.8	3.15
2-(*α*-Hydroxy-*p*-chlorobenzyl)-	265 (193–263)	1.11	415 (390–442)	1.73	About 1,000	~4.2	~2.4	~3.8	1.57
2-(*p*-Chlorobenzyl)-	225 (203–250)	1.01	740 (640–850)	3.33	Close to PD_{50} dose		..	..	3.29
α-Cyclohexyl-*p*-chlorobenzyl alcohol	270 (154–473)	1.20	1,000 (800–1,520)	4.90	1,100	4.90	1.00	4.07	4.07
α-Cyclohexyl-*p*-bromobenzyl alcohol	320 (219–466)	1.19	...	..	.....	..	..	..	..
Phenobarbital Sodium	32 (25–42)	0.126	106 (94–120)	0.416	235[d]	0.925	2.22	7.35	3.31
Mephenesin[e]	...	..	200	1.10	600	3.30	3.0	..	..

[a] The route of administration was intraperitoneal unless otherwise indicated. The values in parentheses are the 95% confidence limits.

[b] The ED_{50} values are for the maximal electroshock seizure (M. E. S.) test unless otherwise indicated.

[c] The curves did not deviate significantly from parallelism.

[d] This value reported by Way, E. L. Leong, *J. Pharmacol. Exptl. Therap.*, **87**, 265(1946).

[e] Values for mephenesin are by Hine, C. H., *et al.*, *ibid.*, **97**, 414(1949).

steric properties is difficult to accept because the difference did not exist in the chloro series. Whereas rapid absorption and elimination were characteristic of the behavior of the *o*-compound in mice in the ED_{50}–PD_{50} dose range, at toxic doses paralysis continued for relatively long periods of time. In rats the duration of paralysis was longer than it was in mice and is referred to subsequently.

In contrast to the methylphenyl isomers, the activity of the chlorophenyl compounds was characterized by a longer time of onset and considerably longer duration of the anticonvulsant property. A possible explanation for this difference is a localization of the compounds in the peritoneal cavity due to a slow rate of solubilization by tissue fluids. The possibility of a difference in metabolic rates may also provide an explanation for the differences in the duration of effects in the two series. The paralyzing potency of members of the chloro series was considerably less than that of the ortho member of the methylphenyl series, but approximately the same as that of the *m*- and *p*-methylphenyl isomers.

Although structure-activity relationships are not too apparent from this study, it may be concluded that the structural changes introduced modify the biological properties of cyclohexanone. The degree to which this occurs is obscured by the fact that the water solubility of the new compounds is markedly less than that of cyclohexanone. A decrease in water solubility frequently results in an enhancement of biological activity (11).

Anticonvulsant.—On a basis of millimoles per kilogram, 2-(*p*-aminophenyl)cyclohexanone, as the hydrochloride, was the most effective anticonvulsant of the compounds studied (see Table II). The lack of stability of the free base and the fact that freshly prepared solutions were soon discolored indicate that this compound itself has serious limitations because of its instability, but suggests investigation of related compounds that might be more stable. Of particular interest, however, is the fact that the anticonvulsant property of this compound at its ED_{50} level continued for more than two hours, indicating a difference in metabolic behavior when compared to other members of the series, for example, the *o*-methylphenyl isomer. The good agreement between the ED_{50} doses of the 2-(*o*-methylphenyl) derivative on a millimole basis (see Table II) when assayed by both the maximal electroshock and pentylenetetrazol methods leads one to suspect that the compound may be acting by preventing seizure spread as well as by elevating the threshold for convulsive discharges. It has been postulated by Goodman, *et al.* (9), that modification of the maximal electroshock convulsion by drugs is accomplished by inhibition of seizure spread, whereas inhibition of both the spread of the seizure and the elevation of threshold occur in modification of the pentylenetetrazol-induced seizure. On the basis of comparative anticonvulsant potencies, it might be assumed that the cyclohexanone derivatives act as anticonvulsant agents by both mechanisms.

Muscle Relaxant.—When the compounds were administered in doses exceeding those producing anticonvulsant effects, but in less than lethal doses, a frequent finding was a marked, reversible flaccid muscle paralysis not unlike that produced by mephenesin. The corneal reflex remained intact. Loss of the righting reflex was preceded by ataxia and paralysis of the hind limbs followed by a stage of excitation characterized by rolling motions and backward arching of the neck. Of the compounds studied, 2-(*p*-aminophenyl)-cyclohexanone caused the most severe excitation prior to the general muscular paralysis. In the methylphenyl series, the ortho-isomer was much more potent than the *m*- and *p*-compounds, although this apparent steric effect was not observed in the chlorophenyl series. The flaccid muscle paralysis resulting from administration of the ortho-compound was conspicuous. In order to compare this action with that of mephenesin, the time of onset and duration of paralysis of the two compounds were determined. Two groups of ten mice each, were given 1.5 PD_{50} doses of mephenesin and the ketone, respectively. The time of onset of paralysis was slightly shorter with mephenesin, whereas the duration of effect from the *o*-methylphenyl compound was somewhat longer. Animals receiving mephenesin did not exhibit the excitatory stage characteristic of the ketone. The corneal reflex remained intact in both groups.

The *o*-isomer was administered to rats in doses as high as 1,000 mg./Kg., at which dose 100% deaths occurred. Six rats received 750 mg./Kg. from which two animals died and paralysis in the remaining persisted for twenty to fifty hours. One rat that had been paralyzed for fifty-two hours with intact corneal reflex was sacrificed and autopsied. The examination "revealed no gross abnormalities," except severe contraction of the bowel which might have resulted from exposure and deprivation of food and water. Sections were made of the vital organs in which "no acute toxic degeneration was seen." Several other animals were autopsied after single doses of the compound from which no evidence of pathology was observed. At 500 mg./Kg. the paralysis lasted for three to four hours and was reversible. The behavior of 2-(*o*-methylphenyl)-cyclohexanone in rats suggests some degree of species specificity in that the compound is not only more toxic in mice, but it may undergo a different metabolic pathway.

Toxicity.—The characteristic toxicity from sublethal doses of the compounds is presented in the discussion of muscle relaxing properties in which reference is made to the excitatory phase of the toxicity. Although this was not an invariable feature of the toxicity pattern, it was observed frequently. When amounts approaching lethal doses were administered, the excitation was often manifest by convulsions, some characterized as "rolling" convulsions and others in which a simultaneous contraction of the muscles of both front and hind limbs occurred. Following these convulsions animals often became highly depressed, from which state recovery sometimes occurred. Respiration usually became labored and abdominal. On the basis of the limited information that the semichronic toxicity experiment provided, it appeared that in general the chronic toxicity of 2-(*o*-methylphenyl)cyclohexanone might be relatively low. The growth curves for the experimental and control groups were similar as were the outward appearances of the animals in both groups. The pathology report revealed little or no damage to the following tissues: heart, lungs, trachea, liver, spleen, kidneys, adrenals, pancreas, thymus, thyroid, esophogus, stomach,

small intestine, cecum, rectum, ovaries, testicles, brain, eyes, skin, and muscle.

Effect of 2-(*o*-Methylphenyl)cyclohexanone on Strychnine Convulsions.—It was of interest to see what effect a member of this series would have on strychnine convulsions. In control mice, 16/20 gave the characteristic strychnine convulsion in three to five minutes with 16/20 fatalities. When *o*-(methylphenyl)cyclohexanone, in approximately PD_{50} doses, was administered twenty minutes prior to the strychnine 20/20 mice convulsed and 18/20 died. The pattern of convulsion was not the same in the two groups. Whereas the control mice in general exhibited the tonic hind leg extensor thrust, it was observed only twice in the experimental group. The pattern in the latter became one of trembling and alternate muscle clonus and relaxation. The behavior suggested an alternate refractoriness and susceptibility as described by Goodman, *et al.* (9), in their discussion of "recurrent seizures following large intravenous doses of metrazol...". Although the ketone had but little influence on the incidence of death, it did postpone death from a range of three to five minutes in the control group and from two to eighty-five minutes (average twenty-six minutes) in the experimental group. In the second experimental group the ortho isomer was administered in a higher dose (0.6 LD_{50}) followed by the strychnine in ten minutes. The ten-minute interval was selected in order to obtain protection at the time of peak anticonvulsant effect. At the time of injection of the strychnine, all mice were paralyzed. The pattern of convulsion was modified, again suggesting a degree of refractoriness to the action of strychnine. The tonic hind leg extensor component of the seizure was absent. An interpretation of the complex convulsant-anticonvulsant relationships cannot be made on such limited data but it appears that the methylphenylcyclohexanone modified the convulsive pattern initiated by strychnine without affecting its lethality. It may be concluded that 2-(*o*-methylphenyl)cyclohexanone, at least to some degree, antagonized the cord effect of strychnine.

In order to provide a basis for comparing relative efficacies of the compounds studied, ratios of anticonvulsant, muscle relaxant, and toxicity data were established (see Table II). These indexes show that 2-(*o*-methylphenyl)cyclohexanone had not only the highest anticonvulsant-toxicity ratio, but likewise the most favorable muscle relaxant-toxicity relationship. Using phenobarbital sodium and mephenesin as reference standards for anticonvulsant and muscle relaxing potency, respectively, 2-(*o*-methylphenyl)cyclohexanone possessed a slightly narrower anticonvulsant but broader muscle relaxant indexes in mice.

CONCLUSIONS

1. The anticonvulsant, muscle relaxing, and toxic properties of a series of cyclohexanone derivatives have been studied in mice.

2. Although, in general, the water solubility of compounds studied is less than that of cyclohexanone, the biological activity is greater.

3. 2-(*p*-Aminophenyl)cyclohexanone was the most active anticonvulsant on a weight basis, whereas 2-(*o*-methylphenyl)cyclohexanone possessed the highest anticonvulsant-toxicity index. The *m*- and *p*-isomers were considerably less active than the *o*-compound. It is postulated that the mode of anticonvulsant action may be due to both elevation of seizure threshold and prevention of seizure spread.

4. In comparison with the methylphenyl series, the action of the chlorophenyl isomers was slower in onset and longer in duration.

5. In amounts exceeding ED_{50} and PD_{50} doses, most of the compounds studied produced varying degrees of a reversible flaccid muscle paralysis. The time of onset and duration of action of 2-(*o*-methylphenyl)cyclohexanone when compared with mephenesin were similar. The muscle relaxant-toxicity index of 2-(*o*-methylphenyl)cyclohexanone was greater than that of mephenesin.

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Statistical Problems Related to the Measurement of Potency of Mydriatic Agents*

By E. G. VAN PROOSDIJ-HARTZEMA

ED_{50} values and potency ratios are often used as if they were constants of the compounds involved. It is well known that these statistics, far from being constant, are highly dependent on the technical details of the pharmacological method. Using the estimation of mydriatic potency as an example, the author shows that the conventional measures of potency also may vary considerably according to the statistical design of the experiments.

IN TWO PREVIOUS PAPERS from this laboratory (1, 2) it was shown that the potency of mydriatic agents in terms of a standard drug (e. g., atropine) is a function of the duration of the observation period. This has since been confirmed by Jageneau and Janssen (3). The object of the present paper is to demonstrate that there may be an additional influence of statistical conventions on the outcome of drug evaluations. To explain both points we subjected the same experimental material to different statistical treatments.

EXPERIMENTAL

Technique.—The experiments were carried out on white mice of an inbred strain weighing 16–19 Gm. Three compounds, atropine, 1,1-diphenyl-3-N-piperidino-butyramide, and 2,2-diphenyl-4-N-hexamethyliminobutyramide were compared with homatropine for their local mydriatic potency. These substances will be referred to hereafter as A, B, and C. The compounds were given as salts in a geometric series of concentrations equimolar with homatropine HBr 0.01, 0.02, 0.04%, etc. They were dissolved in an isotonic boric acid-borax buffer solution (pH 6.5). They were instilled into the left conjunctival sack after which the eyelids were kept open for one minute. By keeping the head of the animal in the correct position the solution could be kept in contact with the eye. The pupil diameter was measured with the method described by Pulewka (4) immediately before 10, 60, 120, 240, and 360 minutes after the application of the drug. The diameters were expressed in arbitrary units. A control series of 140 mice was used; they were given only the buffer solution.

In the pharmacological series about 300 mice were used per compound. Appropriate concentrations of each compound were given to groups of 10 mice. The graphical method of Litchfield and Wilcoxon (5) was used to estimate ED_{50} values and potency ratios with their 95% confidence limits.

Design of the Experiments.—The following designs were used. The algebraic sums were computed of the variations of the pupil diameters of the individual mice over a period of 360 minutes (Design I). The frequency distribution of this statistic was studied in 140 control animals (see Fig. 1). According to the Camp-Meidell Inequality (6),[1] a value above +19 could be considered as a mydriatic response at the 95% probability level.

The variations of the pupil diameters at the separate points of time were also judged by an all-or-none criterion (Design II). The frequency distributions of these statistics are shown in Fig. 2. According to the Camp-Meidell Inequality (6) a value above +4 could be considered as a mydriatic response at the 95% probability level at any point of time.

The same material was subjected to a statistical treatment based on the observation of graded mydriatic effects. The pupil diameters (x) at the different points of time were transformed on a precentage scale (y) by the following equation: $y = 2.2(x-10)$ (Design III). After this transformation the resting pupil diameter was 0 ± 5 and the maximal diameter 100 ± 5. A good linear relationship could be obtained between the log dosages and the probits of the transformed pupil diameters. ED_{50} values with their 95% confidence limits and potency ratios were computed as described above.

RESULTS

The statistical methods described above were applied to the same material, consisting of the outcome of the measurement of pupil diameters in 1200 mice treated with four different drugs. In this way we obtained a complete set of ED_{50} values for each statistical method.

The P. R.'s, in relation to homatropine, are given in Tables I, II, and III. In only one case did the dosage activity curves deviate significantly from parallelism according to the criterion of Litchfield and Wilcoxon (5). In Table II the potency ratio of this case is shown between brackets and without confidence limits.

It will be seen from these tables that the ranking of the activities of the four drugs involved is the same for each statistical method, with the important qualification that in some cases a particular method did

* Received July 8, 1957, from the Nedchem Institute for Pharmaco-Therapeutic Research, van Limburg Stirumstraat 39–43, Amsterdam W., Holland.

Compounds B and C were prepared by the Synthetic Department of the Research Laboratories of Dr. C. Janssen (Beerse, Belgium) (to be published by Paul Janssen, *et al.*).

The able technical assistance of Mrs. A. A. Mens-Boompaal is gratefully acknowledged. The author wishes to thank Miss M. G. J. A. Dijkzeul for the statistical computions.

[1] The Camp-Meidell Inequality states that $P_{n\sigma} \geq \left[1 - \frac{1}{2.25}n^2\right]$, where $P_{n\sigma}$ is the probability that an observation will lie within the interval $\pm 1n\sigma$ from the mean.

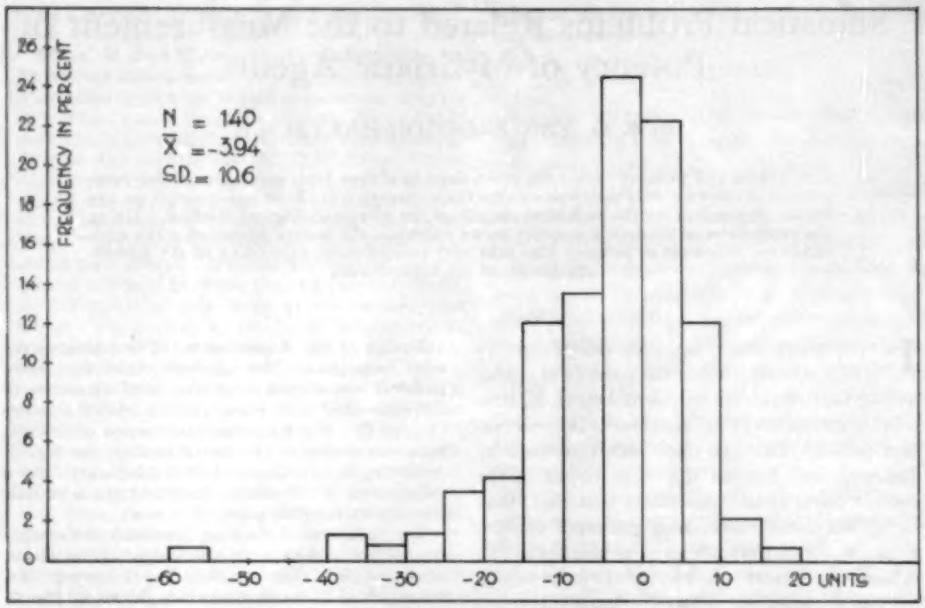

Fig. 1.—The frequency distribution of the algebraic sums of the variations of the pupil diameters in 140 mice over a period of 360 minutes, after the local application of a buffer solution.

TABLE I.—POTENCY RATIOS OF 3 MYDRIATIC AGENTS WITH THEIR 95% CONFIDENCE LIMITS IN RELATION TO HOMATROPINE ACCORDING TO CRITERION I

Compound	Design I 10'–360'
Homatropine	1
A	34.2 (23.1–50.6)
B	34.2 (24.3–48.2)
C	125.0 (95.4–163.8)

not distinguish between drugs which were significantly different according to another criterion. For instance, the dosage activity curves of compounds A and B according to criterion I (see Table I) are identical whereas compound B is about three times more active than A according to criterion III applied after ten minutes (see Table III) [P. R. = 3.5 (2.1 – 6.1)]. If, however, potency ratios of different compounds obtained with different statistical criteria are mutually compared, any desired ranking of the compounds involved can be obtained. One example may suffice because others can easily be obtained by inspection of the tables. The P. R. of compound A according to criterion II applied after 120 minutes is 83 (see Table II), that of compound B according to criterion III after sixty minutes is 46 (see Table III), and that of compound C judged by criterion II after ten minutes is 7.6 (see Table II). This would lead to a ranking of the three compounds in which A is the most potent and C the weakest compound. The true ranking, however, is the opposite.

DISCUSSION

From these data it follows that misleading results can be obtained if potency ratios or ED_{50} values from different sources are compared. The details of the pharmacological method used (strain of the experimental animal, room temperature, background of noise, etc.) or the duration of the observation period are not the only factors which may influence the outcome of an experiment. Even if these sources of variation are eliminated, important differences between the final results may follow the use of different systems of statistical conventions. In my opinion this source of error does not receive the attention which it undoubtedly deserves. This follows quite clearly from even a superficial inspection of the current pharmacological and toxicological reference books. In the majority of these works ED_{50} and P. R. values are used as if they were constants not dependent on the manner in which they were obtained.

As regards the choice of the time interval it should be chosen in accordance with the use to which the drug may be put.

SUMMARY

The results of a detailed investigation of the local mydriatic effect of four drugs in mice were

TABLE II.—POTENCY RATIOS OF 3 MYDRIATIC AGENTS WITH THEIR 95% CONFIDENCE LIMITS IN RELATION TO HOMATROPINE ACCORDING TO CRITERION II

Compound	Design II 10′	Design II 60′	Design II 120′	Design II 240′	Design II 360′
Homatropine	1	1	1	1	1
A	2.2	9.8	83.0	75.0	45.4
	(1.3–3.9)	(6.1–15.7)	(50.0–137.8)	(46.3–121.5)	(29.9–69.0)
B	5.1	14.1	115.8	120.0	91.5
	(3.1–8.4)	(8.6–23.1)	(66.6–201.5)	(80.5–178.8)	(69.3–120.7)
C	7.6	45.9	(463.2)	480.0	380.7
	(4.5–13.0)	(29.4–71.6)		(311.7–739.2)	(262.5–551.9)

TABLE III.—POTENCY RATIOS OF 3 MYDRIATIC AGENTS WITH THEIR 95% CONFIDENCE LIMITS IN RELATION TO HOMATROPINE ACCORDING TO CRITERION III

Compound	Design III 10′	Design III 60′	Design III 120′	Design III 240′	Design III 360′
Homatropine	1	1	1	1	1
A	4.0	41.8	61.9	51.8	42.5
	(2.3–6.9)	(25.7–68.2)	(34.4–111.4)	(28.9–92.7)	(23.4–77.4)
B	11.7	46.0	92.9	131.8	134.2
	(7.6–18.1)	(28.8–73.6)	(54.0–159.7)	(78.0–222.8)	(74.2–242.9)
C	14.2	153.3	361.1	467.7	542.6
	(9.0–22.4)	(92.4–254.5)	(208.7–624.7)	(271.9–804.5)	(310.0–949.5)

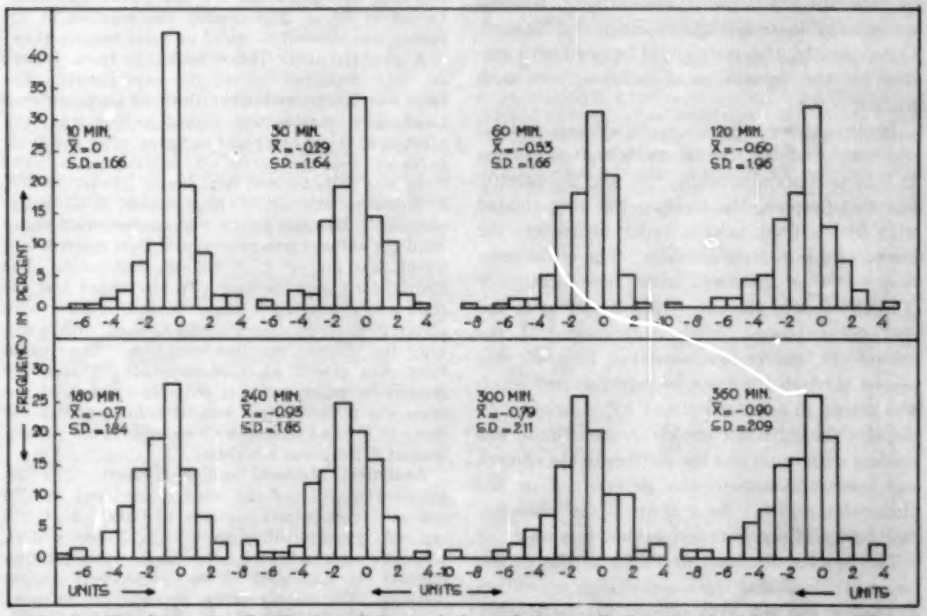

Fig. 2.—The frequency distribution of the variations of the pupil diameters in 140 mice at different intervals after the application of a buffer solution.

used to demonstrate the influence of statistical conventions on the potency ratios. In the author's opinion this source of variation is often neglected.

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Ion Exchange Method for Determination of Caffeine in Presence of Antipyrine and Phenacetin*

By EERO SJÖSTRÖM and LALLI NYKÄNEN

A new method is presented for the determination of caffeine in mixtures containing antipyrine and phenacetin. The mixture is first treated with hydrochloric acid in order to convert the phenacetin into *p*-phenetidine and then extracted with chloroform. After evaporation of the chloroform, the remaining residue containing caffeine and antipyrine is dissolved in water and passed through an ion exchange column filled with a resin in the ferric iron form. Owing to complex formation, antipyrine is quantitatively retained in the column. The caffeine, however, is completely displaced with water at elevated temperature and is spectrophotometrically determined in the ultraviolet region. This procedure has been found to be simple and to give a very satisfactory degree of accuracy.

IN A PRECEDING PAPER (1) complex formation between antipyrine and a cation exchange resin in the iron (III) form was studied. A column filled with the ferric iron resin was found to take up antipyrine quantitatively, whereas under suitable conditions caffeine passed through. Consequently, this resin could be used with success for the separation of caffeine from antipyrine.

In this paper, the above-mentioned method was employed for the determination of caffeine in tablets containing antipyrine and phenacetin. For this purpose, the mixture was first treated with hydrochloric acid in order to convert the phenacetin into *p*-phenetidine. The acidic solution was then extracted with chloroform, only the caffeine and the antipyrine dissolving in the chloroform phase. After evaporation of the chloroform, the residue dissolved in water was passed through the ion exchange column, which was placed in a water bath at 70°. In order to displace the caffeine completely, the column was washed with water and the caffeine in the effluent was spectrophotometrically determined in the ultraviolet region. As a control, the phosphomolybdic acid precipitation method was used.

The results obtained in this and the earlier paper (1) show that the ion exchange separation method combined with ultraviolet spectrophotometry gives very satisfactory accuracy. Since the procedure is quite simple and the resin is capable of taking up large amounts of antipyrine, the new separation method promises to be very useful. It is important from the analytical point of view, because the methods hitherto proposed for the purpose are laborious and more or less inaccurate (2).

* Received May 31, 1957, from the Analytical Laboratory of Lääketehdas Orion Oy, Pharmaceutical Manufacturers, Helsinki, Finland.

EXPERIMENTAL

Preparation of the Resin.—The ion exchange columns were of standard type (3), measuring 22 x 220 mm. and 0.9 x 160 mm., respectively. The columns were placed in a water thermostat maintained at 70 ± 2°, whereas the solution in the funnel was allowed to stand at room temperature.

A strongly acidic cation exchange resin, Dowex 50, was employed for all the experiments. The resin was first ground in the air-dried state, screened to obtain a particle size of 0.15 to 0.30 mm., and allowed to stand overnight in water, after the finest particles were decanted off several times. The resin was then purified with about 200 ml. of 3 *N* hydrochloric acid at 70° in a column of the larger size (22 x 220 mm.). It was washed with water until the effluent was neutral and then treated with about 200 ml. of 1 *N* sodium hydroxide. The hydrochloric acid treatment was repeated and the resin was converted into the ferric iron form with about 10% ferric chloride and washed with water until the effluent was free from iron. The treated resin was stored in a moist state. Before the separation experiments, a suitable amount of the resin was poured into a smaller column (0.9 x 160 mm.) at 70° and washed with water until the absorbance at 272 mμ was less than 0.05.

Analytical Methods and Equipment.—The determinations of caffeine were carried out at 272 mμ at a concentration range of 0.0075 to 0.015 mg./ml., corresponding to E (1%, 1 cm.) = 5180. Daoust's phosphomolybdic acid precipitation method (4) was used as an independent control method. The measurements were carried out using a Zeiss Spectrophotometer PMQ II fitted with matched 1-cm. absorption cells.

PROCEDURE

Weigh a suitable aliquot of the powdered tablets corresponding to about 25–50 mg. of caffeine. Add 20 ml. of 0.5 *N* hydrochloric acid and allow to boil for one hour under reflux. After cooling extract the solution three times with 20 ml. of chloroform and evaporate the chloroform phase in a water bath under reduced pressure. Dissolve the dry residue in about 10 ml. of water, filter if necessary, and

pour through a column at 70° filled with the purified ferric iron resin. Regulate the flow-rate with the stopcock to about 1 ml./min. during the sorption step and at the beginning of the washing with the first 25–50 ml. Then the flow rate can be increased to about 4 ml./min. Collect the filtrate in a 250-ml. volumetric flask and wash the column with water to obtain an effluent volume nearly to the mark with water. Dilute the effluent to a suitable concentration range, measure at 272 mμ against water, and calculate the caffeine content from the absorbance.

Since a dark brown-colored zone is formed when antipyrine comes into contact with the resin, the saturation can be visually followed. As the antipyrine gradually moves downward in the column when water is passed through it, the capacity of the resin also depends upon the amount of water used. When 250 mg. of antipyrine is used, the same column may be used for two separations. Since large amounts of regeneration solution are required to elute antipyrine completely from the column, it is best, to fill the column with a fresh resin after saturation.

Gas bubbles frequently appear in the upper layer of the resin bed, obstructing the flow. These can be removed with a fine glass-staff.

RESULTS

The analysis data obtained with different tablets are summarized in Table I. Samples 1 to 5 were commercial preparations; whereas samples 6 to 9 were prepared from the individual compounds in an ordinary tablet base (starch and talcum). As can be seen, the agreement between the theoretical and observed values is highly satisfactory. Furthermore, the data obtained by the ultraviolet analysis and by the phosphomolybdic acid precipitation method agree well.

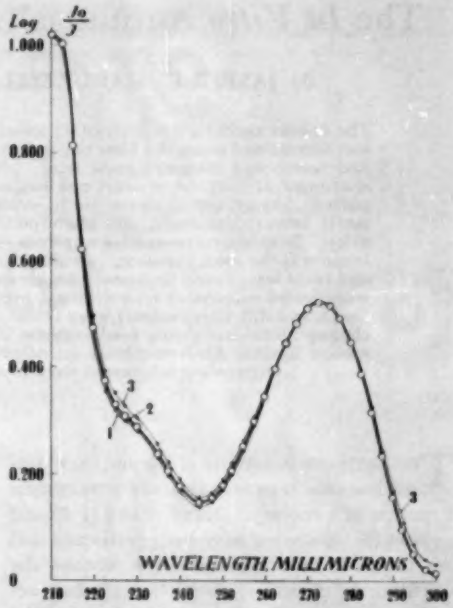

Fig. 1.—Ultraviolet absorption spectrum of caffeine after ion exchange separation (curve 1): caffeine standard solution (0.01 mg./ml.) (curve 2); diluted effluent after separation of caffeine (50.2 mg.) from antipyrine (251.8 mg.). Theoretical concentration of caffeine 0.0100 mg./ml. (curve 3); diluted effluent after separation of caffeine (50.5 mg.) from antipyrine (250.2 mg.) and phenacetin (125.0 mg.). Theoretical concentration of caffeine 0.0101 mg./ml.

TABLE I.—SEPARATION OF CAFFEINE FROM ANTIPYRINE AND PHENACETIN

Sample No.	Compounds present: Antipyrine, mg.	Phenacetin, mg.	Caffeine (Anhydrous), mg.	Caffeine Found UV-Analysis, mg.	Error, %	Caffeine Found Daoust's Method, mg.	Error, %	Deviations Between Methods Used, %
1	250	125	50	50.8	+1.6	47.2	−5.6	7.2
2	250	125	50	49.2	−1.6	47.9	−4.2	2.6
3	250	125	50	52.2	+4.5	49.2	−1.5	6.0
4	250	25	91.5	91.4	−0.1	90.8	−0.8	0.9
5	250	25	91.5	90.6	−0.9	90.6	−0.9	0.0
6	250	125	25.6	26.65	+4.1	25.6	±0.0	4.1
7	250	125	49.9	49.4	−1.2	49.7	−0.3	1.0
8	250	125	52.2	51.4	−1.5	50.3	−3.6	2.1
9	250	125	150.1	152.1	+1.4	151.0	+0.6	0.8

In order to examine the purity of the caffeine after the ion exchange separation, the ultraviolet absorption spectrum of the effluent was measured. As can be seen from Fig. 1, the spectra of the effluent after the separation of caffeine and antipyrine (curve 2) and caffeine, antipyrine, and phenacetin (curve 3), coincide nearly completely with the spectrum of pure caffeine (curve 1). From this experiment it may thus be concluded that the ion exchange method results in an excellent separation of the components.

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The *In Vitro* Antifungal Activity of Essential Oils*

By JASPER C. MARUZZELLA and LAURENCE LIGUORI

The *in vitro* antifungal activity of 92 volatile oils, 12 terpeneless oils, and 3 fixed oils was ascertained using the filter paper disk method. The oils were tested against 18 pathogenic and nonpathogenic fungi. Ninety volatile oils were found to possess antifungal activity on at least one organism. Origanum (red), lemongrass (imported), thyme (red), sweet birch, savory select, coriander, sassafras, cinnamon, laurel leaves (distilled), and chenopodium exhibited the greatest antifungal activity. *Streptomyces venezuelae* was most susceptible to the volatile oils while *Candida krusei* was the most resistant. Among the terpeneless oils, cinnamon, caraway, dill, and anise were found to possess the greatest antifungal activity. The fixed oils used were found to possess no antifungal properties. Oils of chenopodium, origanum (red), and dill (terpeneless) were found to be very effective against *Ustilago avenae;* chenopodium and thyme (red) against *Trichophyton mentagrophytes;* and oil of cinnamon against *Epidermophyton interdigitale.* More than 90% of the volatile and terpeneless oils tested were found to produce fungicidal effects.

THE ANTIFUNGAL activity of oils and their constituents has been investigated for more than a quarter of a century. Many *in vivo* (1–5) and *in vitro* (6–10) studies have been performed and yet these oils have apparently not received the attention in clinical application that their activity warrants. Furthermore some beneficial effects have been obtained with oils against plant fungus diseases (11–13). The present investigation was undertaken to re-evaluate the antifungal activity of essential oils using the *in vitro* filter paper disk method exclusively.

MATERIALS AND METHODS

The detection of the antifungal activity of various volatile, terpeneless, and fixed oils was made by observing their effects on growing cultures of 18 organisms both pathogenic and nonpathogenic. The organisms used were: *Candida albicans, Candida krusei, Candida tropicalis, Saccharomyces cerevisiae, Rhizopus nigricans, Cryptococcus neoformans, Cryptococcus rhodobenhani, Aspergillus fumigatus, Aspergillus niger, Helminthosporium sativum, Alternaria solani, Mucor mucedo, Nigrospora panici, Penicillium digitatum, Ustilago avenae, Trichophyton mentagrophytes, Epidermophyton interdigitale,* and the moldlike bacterium *Streptomyces venezuelae.* The method used for detecting the presence or absence of antifungal activity was similar to the method of Vincent's (14) qualitative filter paper disk diffusion plate method on bacteria. In this method small sterile disks made of paper (1/4 inch in diameter) were thoroughly moistened with the oil to be tested and placed on Sabauraud's maltose agar plates which had been previously seeded with 2 cc. of the organism in broth culture. All dishes were conducted in triplicate with two or three disks per dish. The measurement of each zone of inhibition represents a mean value of a minimum of six recordings. All of the organisms were grown in Sabauraud's maltose broth for seventy-two hours with the exception of *T. mentagrophytes, E. interdigitale,* and *U. avenae* which were cultivated for one week. All plates were incubated at room temperature for forty-eight hours with the exception of the above three-mentioned organisms which were incubated for one week at room temperature.

The presence of a zone of inhibition of any size surrounding the paper disks indicated antifungal activity. The zones of inhibition were measured with a metric ruler with the aid of an illuminated Quebec colony counter. All of the zones of inhibition were tested to ascertain whether the activity was fungicidal or fungistatic. This was performed by making transfers from the clear zones into Sabauraud's maltose broth and incubating for three days. No growth in the broth tubes after incubation was indicative of fungicidal activity. Growth in the tubes indicated fungistatic activity.

As the oils arrived at the laboratory they were placed into sterile bottles and tested for sterility by streaking both nutrient agar and Sabauraud's agar slants. After incubation at room temperature for one week or more the tubes were examined for the presence or absence of growth. All oils were found to be free of microorganisms.

RESULTS AND DISCUSSION

Of the 92 volatile oils tested, 90 were found to exhibit antifungal activity on at least one of the 15 organisms employed. Table I lists these volatile oils with their antifungal activity and zones of inhibition. It may be observed from Table I that the following oils show the greatest sum total of zones of inhibition in this order: origanum (red), lemongrass (imported), thyme (red), sweet birch, savory select, coriander, sassafras, cinnamon, laurel leaves (distilled), and chenopodium. The effects of the volatile oils against each organism were found to be of the following order (figures indicate the sum of the zones of inhibition caused by the oils, arranged in descending order): *Streptomyces venezuelae,* 865 mm.; *Aspergillus niger,* 844 mm.; *Aspergillus fumigatus,* 797 mm.; *Mucor mucedo,* 781 mm.;

* Received June 11, 1957 from the Department of Biology, Long Island University, Brooklyn, N. Y.
This paper is based upon a thesis submitted to the Graduate School of Long Island University by Laurence Liguori in partial fulfillment of the requirements for the degree of Master of Science.

TABLE I.—INHIBITORY ACTIVITY OF VOLATILE OILS

Oils	Zone of Inhibition, mm.[a]															
	Saccharomyces cerevisiae	*Candida krusei*	*Candida tropicalis*	*Candida albicans*	*Streptomyces venezuelae*	*Rhizopus nigricans*	*Cryptococcus neoformans*	*Aspergillus fumigatus*	*Helminthosporium sativum*	*Alternaria solani*	*Aspergillus niger*	*Cryptococcus rhodochrous*	*Mucor mucedo*	*Nigrospora panici*	*Penicillium digitatum*	Sum[b]
Amber, Rect.	0[c]	0	2S[d]	2S	4	1	2S	2S	2	3	3	2S	2S	2	3	30
Anethol	5	0	4S	5	7	5	8	17	12	7	15	10	10	7	14	126
Angelica Root	12	2	2S	3	7	0	11	3	3	11	3	2	3	7	4	81
Anise	0	0	2	2	7	3S	9	9	10	9	13	0	6	12	15	97
Balsam Peru	3	0	6	5	12	3	4S	7	4	5	7	5S	8S	6S	6S	81
Balsam Tolu	4	1	6	7	15	6	7	9	7	9	14	8	11	10	7	121
Basil, Sweet, Amer.	19	0	0	11	14	13	15	17	19	10	17	15	16	10	13	162
Bay	8	5	7	5	14	8	13	12	9	12	14	12	8	9	12	148
Bergamot	5	3S	3S	5	8	3	3	4	4	5	4	3S	5	5	6	66
Birch Tar, Rect.	6	3	5	4	12	5	8	11	10	7	13	11	3	6	11	115
Cajuput	14	6	8	6	16	15	9	13	9	8	7	10S	11	12	7	151
Calamus	0	0	0	1S	4	1S	0	3	0	2	4S	0	3	2	2S	22
Cananga	0	0	0	0	2	0	0	0	0	2	2	0	0	0	0	6
Caraway	0	0	0	0	7	6	14	10	12	15	13	0	9	15	7	108
Cardamom	4	0	2	1	15	3	3	4	5	5	7	3	6	6	4	68
Cascarilla	0	0	0	0	5	1	0	1	0	0	0	0	3	0	0	10
Cedar Leaf	3	3	2S	5	8	3	5	5	4	3	5	4	6	4	3	63
Cedar Wood	0	0	0	0	3	0	0	0	0	0	0	0	0	0	0	3
Celery Seed	1	0	0	1S	2	0	3	1S	4	2	3	1S	2S	2	0	20
Chenopodium	8	5	12	9	8	10	13	10	20	20	25	0	9	8	10	167
Cinnamon	6	4	8	15	15	7	18	19	15	12	16	13	12	15	17	192
Citronella	2	0	3	6S	4	1S	2S	6	2	6	7	7	6	6	5	63
Clove	5	5	3	3	9	7	8	10	7	9	10	13S	7	8	9	113
Mountain Laurel	5	4	4	6	11	9	12	13	8	11	9	13	10	12	10	137
Myrcia	6	5	11	7	8	7	12	14	10	2	18	9	14	9	14	146
Myrrh	0	0	1S	2	6	3	4	3	2	3	1S	1S	2S	2S	2	32
Neroli	4	0	0	8	9	6	6	5	9	9	9	6	9	5	9	94
Niaouli	7	5	8	6	10	7	10	20	12	11	22	11	11	7	8	155
Orange, Sweet	18	5	4	5	5	0	6	8	7	13	4	4	4	6	7	96
Origanum Red	26	20	24	24	11	7	20	22	19	20	26	27	23	26	23	318
Parsley	0	0	0	1	5	0	2S	2S	0	0	0	3	2S	0	0	15
Patchouly	0	0	0	0	2S	0	0	0	0	0	0	0	2S	0	0	4
Pennyroyal, European	6	0	4	3	5	7	10	15	23	14	14	0	13	14	15	143
Pepper, black	0	0	0	0	2S	0	0	0	0	0	0	0	0	0	0	2
Peppermint	2S	1	3	4	3	7	3	4	7	0	6	2	8	8	8	66
Petitgrain, Paraguay	2	1	4	5	10	7	10	9	8	8	7	6	8	5	4	94
Pimento	5	4	6	5	10	7	8	9	9	11	10	13	9	9	9	124
Fennel	3	0	0	0	10	3	0	12	6	6	15	20	10	7	7	99
Fir Needle, Canadian	7	0	2S	0	4	0	7	0	1	3	0	0	0	3	0	27
Fleabane, American	0	0	0	0	0	0	2S	0	0	0	0	0	4	0	0	6
Galangal	0	0	2	3	10	4	0	6	3	4	2	3	5	6	6	54
Geranium, Algerian	0	0	4	4	6	3	0	15	8	8	9	5	8	8	8	93
Ginger	0	0	0	0	2S	0	2S	1	2S	1	0	1S	2S	2S	0	13
Hemlock	4	0	1	0	2S	3	3	3	3	7	0	2	2	2	2	34
Juniper Berries	0	0	0	0	0	6	11	13	0	2	6	6	0	0	0	44
Juniper Tar	2	0	1	1	8	0	3	2S	3	8	6	4	4	2	7	51
Laurel Leaves, Dist.	11	0	6	6	20	21	22	15	14	6	13	15	19	12	7	172
Lavandin, French	9	0	3	9	22	14	13	7	7	9	21	10	9	7	3	143
Lavender	3	2	6	7	15	8	0	7	7	0	8	7	10	5	3	88
Lemon	7	3	1S	2S	11	0	5	9	16	6	9	4	11	10	11	111
Lemongrass, Imported	20	0	0	0	20	7	0	30	25	35	35	28	35	27	13	275
Lime, Dist., natural, West Indian	2	0	3	6	11	0	12	7	2S	3	2S	4	6	9	5	72

TABLE I.—(*Continued*)

Oils	Zone of Inhibition, mm.[a]															
	Saccharomyces cerevisiae	*Candida krusei*	*Candida tropicalis*	*Candida albicans*	*Streptomyces venezuelae*	*Rhizopus nigricans*	*Cryptococcus neoformans*	*Aspergillus fumigatus*	*Helmirthosporium sativum*	*Alternaria solani*	*Aspergillus niger*	*Cryptococcus rhodobenhani*	*Mucor macedo*	*Nigrospora panici*	*Penicillium digitatum*	mm[b]
Lime, Exp., natural, West Indian	6	2	4	4	6	0	4	2S	2S	3	2S	2S	5	2S	2	46
Linaloe Wood	8	5	5	0	22	10	10	12	15	7	12	14	13	9	15	157
Mace, Dist.	6	0	2	3	7	0	4	6	2	3	10	2	6	3	0	54
Majoram, Sweet	4	5	8	7	14	5	12	12	6	12	11	9	11	10	8	134
Coriander	7	0	9	5	11	13	5	21	11	18	21	20	25	28	14	208
Cubeb	0	0	0	0	5	10	1	0	0	0	0	0	2	0	0	18
Cypress	9	2	3	2S	13	2	6	4S	6	6	5	3	4	5	5	75
Dill Seed	3	0	0	5	16	15	15	10	13	11	13	0	10	18	11	140
Dwarf Pine Needle	0	0	0	0	2S	0	0	0	0	0	0	0	0	0	0	2
Eucalyptol	5	5	6	5	12	10	6	6	7	4	0	2	0	0	0	66
Eucalyptus	8	5	3	5	13	3	8	13	9	10	7	7	9	10	4	114
Pimento Leaf	7	0	6	7	13	15	8	19	6	10	13	0	12	10	11	137
Pine Needle	5	2S	2S	3	5	5	4	3	4	5	6	3	8	5	3	63
Pine Needle, Siberian	5	0	0	0	5	2S	11	5	3	17	9	0	0	14	6	77
Pinus Sylvestris	18	5	10	5	7	5	6	9	12	8	12	14	15	12	10	148
Rosemary	7	1	2S	2S	10	6	6	6	9	7	3	6	5	5	6	81
Rue	1	0	2	3	17	3	11	8	8	12	15	4	2	9	5	100
Sage, Grecian	7	1	2	2	40	0	5	5	6	7	9	3	9	6	2	104
Sage, Spanish	13	1	7	6	22	6	8	6	6	11	11	6	5	11	6	125
Sandalwood	0	0	0	0	3	0	0	1	0	0	0	0	1	0	0	5
Sassafras	6	4	5	3	8	12	10	14	17	25	16	20	20	15	27	202
Savin	3	2	2S	2S	15	5	3	4	5	5	7	3S	7	4	4	71
Savory, Select	11	0	8	5	24	7	0	18	12	20	29	13	29	30	35	241
Spearmint, Mohawk	10	3	10	5	13	8	9	16	9	13	12	8	15	18	13	162
Spike Lavender	6	3	4	8	13	14	13	9	11	8	12	14	12	8	4	139
Styrax	10	4	4	5	11	8	13	15	11	11	12	9	9	12	13	147
Sweet Birch	4	4	3S	5	10	10	9	13	10	30	30	30	40	11	35	244
Tansy	4	3	4	4	13	7	8	10	7	7	7	7	8	6	7	102
Tarragon	5	2	3	5	6	3	2	20	5	12	7	5	4	20	18	117
Terpineol	6	3	4	9	7	7	11	20	11	4	13	7	11	12	10	135
Thuja occidentalis	10	0	7	5	5	11	11	18	12	10	12	4	12	15	15	147
Thyme, red	16	13	12	14	21	14	25	21	20	22	22	14	22	16	17	269
Turpentine	5	2S	3	1	6	7	5	6	5	0	9	2	11	7	2	71
Verbana, comp.	6	3	6	10	17	3	19	11	10	11	11	12	19	10	6	154
Vetivert, Haitian	0	0	0	0	3	0	0	0	0	0	0	0	2	0	0	5
Wintergreen	4	3	3	0	6	9	10	9	20	12	25	7	25	7	6	146
Wormwood	0	0	0	0	3	0	3	0	2	0	6	1	6	0	4	25
Ylang-Ylang	2S	2S	2S	2S	5	0	3	6	3	0	1S	4S	2S	1	1	35

[a] Measurement from disk edge to zone edge.
[b] Sum of zones of inhibition for each oil.
[c] Zone of inhibition absent.
[d] S = fungistatic activity, the absence of S indicated fungicidal activity. Many of the volatile oils were generously supplied by Florasynth Laboratories, Inc., New York City.

Nigrospora panici, 707 mm.; *Alternaria solani*, 703 mm.; *Penicillium digitatum*, 661 mm.; *Helminthosporium sativum*, 654 mm.; *Cryptococcus neoformans*, 622 mm.; *Cryptococcus rhodobenhani*, 578 mm.; *Saccharomyces cerevisiae*, 428 mm.; *Rhizopus nigricans*, 472 mm.; *Candida albicans*, 357 mm.; *Candida tropicalis*, 325 mm.; *Candida krusei*, 170 mm. Thus, *Streptomyces venezuelae* was the most vulnerable organism while *Candida krusei* was the most resistant. Those volatile oils which possessed no antifungal activity were orris root and rhodium (ordinary).

The effect of 12 terpeneless oils on fungi may be noted in Table II. The terpeneless oils of cinna-

mon, caraway, dill, and anise exhibited the greatest antifungal activity. The most susceptible organism was found to be *Helminthosporium sativum* while the most resistant was *Candida krusei*. By comparing Tables I and II the following may be observed: terpeneless oils of anise, caraway, and sweet orange possess greater antifungal activity than the corresponding volatile oil; volatile oils of eucalyptus petitgrain, spearmint, and bergamot possess greater antifungal activity than their terpeneless oil; and the terpeneless oils of bay, cinnamon, and peppermint seem to possess about the same antifungal property as the corresponding volatile oil.

Less than 10% of the volatile oils listed in Table I and less than 10% of the terpeneless oils listed in Table II were found to be fungistatic while the remaining proved to be fungicidal.

The effect of several oils (taken from Tables I and II) possessing a more powerful antifungal activity were tested against *U. avenae*, *T. mentagrophytes*, and *E. interdigitale*. These results may be found in Table III. The oils of chenopodium, origanum (red), and dill (terpeneless) were very effective against *U. avenae;* chenopodium and thyme (red) were effective against *T. mentagrophytes;* and cinnamon oil was very effective against *E. interdigitale*.

The three fixed oils, castor, olive, and mineral (white) were found to possess no antifungal activity when tested against all of the organisms listed in Table I.

Heretofore apparently no work has been performed on the rather powerful antifungal activity of the volatile oils of origanum (red), lemongrass (imported), sweet birch, and savory select. These oils might possibly be incorporated into antifungal medicaments with great efficacy. Furthermore, it is interesting to note that the antifungal activity of volatile oils does not always parallel its antibacterial activity. Using the identical method that was employed in this investigation, Maruzzella and Lichtenstein (15) reported eucalyptus, birch tar, cinnamon, balsam of tolu, and cedar leaf to possess the greatest antibacterial activity of the 110 volatile oils they

TABLE II.—INHIBITORY ACTIVITY OF TERPENELESS OILS

	Zone of Inhibition, mm.[a]														
Oils	*Saccharomyces cerevisiae*	*Candida krusei*	*Candida tropicalis*	*Candida albicans*	*Streptomyces venezuelae*	*Rhizopus nigricans*	*Cryptococcus neoformans*	*Aspergillus fumigatus*	*Helminthosporium sativum*	*Alternaria solani*	*Aspergillus niger*	*Cryptococcus rhodochrous*	*Mucor mucedo*	*Nigrospora panici*	*Penicillium digitatum*	Sum
Anise	/	0[c]	5	0	3	2	2	26	27	14	28	16	20	7	27	184
Bay	9	0	4	0	13	10	7	6	14	12	15	7	7	12	14	130
Bergamot	4	0	0	0	6	2	6	2S[d]	1S	3	2	4	6	3S	0	39
Caraway	9	0	6	7	11	4	11	14	20	28	25	20	15	14	11	195
Cinnamon	24	4	9	13	15	7	17	19	15	15S	16	9	11	14	12	200
Dill	3	0	8	10	7	7	3	21	23	20	27	20	7	25	13	194
Eucalyptus	1	0	0	9	6	0	6	7	2	4	0	4	7	3S	4	53
Mandarin	5	0	2S	0	7	2S	10	11	22	7	12	7	3	6	7	101
Peppermint	4	0	2S	0	4	6	7	7	5	7	7	7	5	7	6	74
Petitgrain	7	3S	0	0	6	3	7	5	2	5	2	2	4	4	1S	51
Spearmint	3	0	4	6	7	3	7	12	20	10	16	11	5	15	14	133
Sweet Orange	5	0	6	10	9	2	4	16	17	20	16	8	5	11	11	140

[a] Measurement from disk edge to zone edge.
[b] Sum of zones of inhibition for each oil.
[c] Zone of inhibition absent.
[d] S = fungistatic activity, absence of S indicates fungicidal activity.

TABLE III.—INHIBITORY ACTIVITY OF OILS

	Zone of Inhibition, mm.[a]		
Oils	*Trichophyton mentagrophytes*	*Epidermophyton interdigitale*	*Ustilago avenae*
Bay	30	6	40
Caraway, terpeneless	45	10	[b]
Chenopodium	60	6	45
Cinnamon	35	25	23
Cloves	25	5	35
Dill, terpeneless	45	5	45
Origanum, red	10	8	45
Sassafras	35	3	..
Sweet Birch	10	0	..
Thyme, red	60	6[c]	40

[a] Measurement from disk edge to zone edge.
[b] Not tested.
[c] Zone of inhibition absent.

tested. The negative results obtained in this investigation with fixed oils do not agree with the work of Martin and Solmon (11) who found that emulsions of olive and castor oil possessed fungicidal properties. However, it has also been reported that castor oil was not fungicidal (13) and that fixed oils possess little if any fungicidal activity (16, 17).

The mode of action of the antifungal activity of volatile oils is not known. However, according to Owens (18), the quinones (found in essential oils) seem to be highly inhibitory for both malt amylase and catalase of some fungi. Recently Gal'perin and Dunaeva (19, 20) reported that some volatile oils affect dehydrogenase activity of paramecia and helminths. A similar mechanism of action may exist in fungi but further work must be performed to substantiate this.

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Nonenzymatic Destruction of Thiamine

A Chromatographic Study of the Degradation Products*

By W. J. LHOEST,† L. W. BUSSE, and C. A. BAUMANN

A paper chromatographic method was developed for separating and staining the degradation products of thiamine. A study of the behavior of this vitamin as a function of pH showed a progressive formation of the carbinol form of thiamine, thiochrome, probably thiamine disulfide, two pyrimidine derivatives, and two still unknown products. The same method proved satisfactory for the identification and isolation of "glucothiamine," a product which is formed when thiamine is heated in presence of glucose.

THAT THIAMINE TENDS to be unstable was recognized almost immediately after the existence of "vitamin B" was discovered, yet relatively little effort has been expended in a study of its degradation products, except for those that arise on exposure to bisulfite: the characterization of the cleavage products established the formula of the vitamin itself (1). In foods or vitamin concentrates, however, destruction of thiamine is much more likely to occur as a result of heat, alkali, oxidizing agents, inorganic salts (2), heating with glucose (3), or exposure to flavonoids or phenols (4).

The present study deals with products that arise from the exposure of thiamine to alkali or glucose.

METHODS

Solutions of thiamine were held at a series of pH's for a period of two to sixty minutes, and aliquots fractionated by ascending chromatography on Whatman No. 1 filter paper, in chambers made of suitable glass frames surrounded by airtight polyvinyl chloride plastic bags. The solvent systems and specific stains used are described below. Whenever possible given spots were compared with known substances for R_f, reactivity with appropriate reagents, fluorescence under ultraviolet light at 366 mμ (Mineralight Long Wave U. V. Model SL 3660) and absorption spectrum taken on a Cary recording spectrophotometer model 11.

EXPERIMENTAL

Solvent Systems and Detection.—To provide a complex mixture of degradation products for use in a survey of possible solvent systems, a 10% solution of thiamine in 1 *N* KOH was boiled for thirty minutes. One-tenth cc. aliquots of this solution were placed on filter paper in the usual manner and chromatograms developed with the solvent combinations listed in Table I.

The mixtures that appeared most satisfactory were *n*-butanol-acetic acid-water, 4/1/5 and *n*-butanol-ethanol-water, 2/1/1. For the separation of very complex mixtures, a succession of these solvent combinations in a two dimensional system appeared best. For the simpler mixtures following only mild degradation of thiamine, separation was satisfactory with monodimensional chromatography.

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TABLE I.—SOLVENT SYSTEMS USED FOR CHROMATOGRAPHY

n-Butanol	2[a]	Ethanol	1	Water 1
n-Butanol	1	Ethanol	2	Water 4
n-Butanol	1	Ethanol	1	Water 1
n-Butanol	4	Glacial acetic ac		Water 5
Isoamyl alcohol	5	Pyridine	1	Water 3
Phenol, 80 Gm.			5	Water to 100 cc.

[a] Most of these solvents were simple mixtures. Some of them, however, produced two different layers saturated with each other. In this case the organic phase was always used for the chromatography itself while the aqueous layer remained for saturating the chamber.

Six chromatograms were generally run together each of them being subsequently treated in a different way. Table II shows the reagents which were used for the detection of the degradation products.

The most useful reagents for this purpose proved to be ammoniacal silver nitrate (Tollens), at room temperature and at 80° bromphenol blue/silver nitrate before and after washing, potassium ferricyanide, and sodium nitroprusside/sodium cyanide.

Effect of pH on the Chromatographic Behavior of Thiamine.—A 10% aqueous solution of thiamine hydrochloride (pH 2.4) was divided into 12 fractions. Increasing amounts of 9 *N* NaOH were added to these solutions and the following series of pH values were obtained: 2.4, 4.1, 4.9, 6.4, 7.8, 8.5, 9.0, 9.5, 10.0, 11.2, 13.0. Immediately after their preparation, 0.1-cc. samples of each of these solutions were put on six different papers and run concurrently for fifteen hours with the solvent system *n*-butanol-ethanol-water, 2/1/1. NaOH, NaCl, thiamine (free base), and thiochrome were run as reference compounds. The results of these experiments are shown in Fig. 1. Direct examination of the dried chromatogram under ultraviolet light showed a row of bright blue fluorescent spots (*A*) all having the R_f of thiochrome (0.58). Repeated chromatography with a different solvent system confirmed this similarity. Under the conditions described, thiochrome was formed in very small amounts at pH 7.8, reached maximum intensity at pH 8.5 and 9.0, and decreased progressively from pH 9.5 to pH 13.0, where the spot

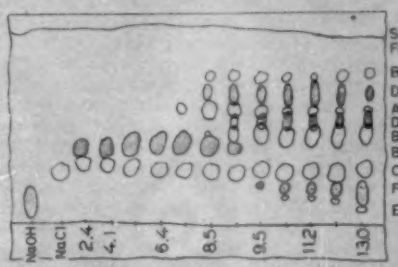

Fig. 1.—Effect of pH on the chromatographic behavior of thiamine solutions. Solvent system: *n*-butanol-ethanol-water 2/1/1. The diagram is a composite: spraying with ammoniacal silver nitrate revealed spots B, B_1, C, E; spraying with potassium ferricyanide revealed B_1 and B_1'; spraying with bromphenol blue/ silver nitrate revealed D, D', and F; sodium nitroprusside gave a red coloration with spot D in presence of sodium cyanide while F also reacted with normal acid-base indicators; spots A and B were seen under ultraviolet light. R_f values are in parentheses.

TABLE II.—SPRAY REAGENTS USED FOR DETECTING DEGRADATION PRODUCTS

Reagent	Detected Products	Method of Application
Potassium permanganate (5)	Almost any organic compound	Spraying
Ammoniacal silver nitrate (Tollens) (6)	Reducing substances and many other organic compounds	Spraying; drying at room temperature or in an oven at 80°
Bromphenol blue/silver nitrate (7)	Halides (pink); purines and pyrimidines (blue)	Spraying; drying at room temperature and washing by immersion in dist. water
Aniline phthalate (8)	Glucosamines; reducing compounds; furfural; uronic acids	Spraying; heating at 110°
p-Dimethylamino-benzaldehyde (Ehrlich) (9)	Glucosamines	Spraying; heating at 37° for 2 hr.
Potassium ferricyanide (10)	Thiamine	Spraying; observation in ultraviolet light
2,6-Dichlorophenol indophenol (Tillmans) (11)	Glucosamines; reducing compounds	Spraying
2,4-Dinitrophenyl hydrazine (12)	Furfurals	Spraying; observation in ultraviolet light
Diazo reaction (13)	Thiamine	Spraying
N-Ethyl maleinimide (14)	—SH groups	Spraying with soln I; drying in air for 15 min.; spraying with soln. II
Ninhydrin (15)	$-NH_2$ groups in aliphatic chains	Spraying; drying in darkness for 24 hr.
Triphenyl tetrazolium chloride (16)	Reducing sugars	Spraying and keeping in moist atm. at 40° for 2 hr.; washing with water
Sodium nitroprusside/sodium cyanide (17)	—SH groups; —S—S— groups	Spraying with soln. I (—SH); dipping in soln. II (—S—S—)

was scarcely visible. Spraying the paper with ferricyanide reagent (10) converted thiamine to thiochrome, and as a result two different rows of spots appeared, the first in the acidic region at R_f 0.40 (B_1) and the second in the basic region with R_f values of 0.46 (B_1'). A number of considerations led to the conclusion that spot B_1 (R_f, 0.40) was due to thiamine chloride, while B_1' (R_f, 0.46) was due to the carbinol form of thiamine. Spraying another paper with the ammoniacal silver nitrate reagent (6) produced a brown coloration with all the spots having an R_f of 0.40, while the row of spots at R_f 0.46 remained uncolored. Furthermore, at pH 8.5, spot B_1' appeared while that of B_1 decreased in intensity slightly. At pH 9.0 both spots were approximately of the same size and at pH 9.5 all B_1 seemed to have been converted to B_1'. The titration curve of thiamine (18) indicated that the reaction occurring at pH 9.5 was the conversion of the free thiamine to the carbinol form. Both the chloride and carbinol forms of thiamine would be expected to give the thiochrome reaction, but the replacement of the chlorine atom in the thiazole ring by an hydroxyl group would prevent the reaction with silver nitrate (Tollens). The presence of the lower spot "C" (R_f 0.28) which was given by sodium chloride either pure or mixed with decreasing amounts of hydrochloric acid indicated that thiamine was ionized under the conditions of the separation and that the free base (B_1) migrated independently on the paper. Spot "F," present in increasing amounts from pH 9.0 to 13.0, appeared to be sodium hydroxide. This was shown by comparison with a standard and by spraying with acid-base indicators. The presence of sodium hydroxide above pH 9.5 suggested by the results of the staining procedure was also indicated by the characteristics of the titration curve (18). The upper spot "B" (R_f 0.76) and the lower one "E" (R_f 0.14) are still unknown. "B" gave a blue fluorescence under ultraviolet light and a brown color with Tollens reagent. It was always present in the pH range 8–14. On the other hand, "E" which appeared from pH 10.0 to 13.0 might bear some relation to the opening of the thiazole ring which occurs above pH 9.0.

In the next experiment the chromatogram was sprayed with the bromphenol blue-silver nitrate reagent which has been used for the detection of purines and pyrimidines (7). After generous washing by dipping in distilled water, there remained on the chromatogram two rows of intense blue spots which were not visible by the other staining procedures. These spots were designated "D" and "D'." Their respective R_f's were 0.64 and 0.52, and they were separated by spot "A" of thiochrome. Further information on the nature of "D" was obtained by spraying the paper with reagents for —SH and —S—S— groups. Copious wetting of the paper with nitroprusside reagent did not give any evidence of —SH groups. However, further spraying with the solution of sodium cyanide showed a very bright red coloration with spot "D," suggesting the presence of an —S—S— bridge. The corresponding spot was then located by its R_f on a chromatogram that had not been sprayed, and this area cut out and eluted with water. Three fractions of this solution were adjusted to pH 2.35, 7.10, and 11.75, respectively, and run on the spectrophotometer. The ultraviolet absorption curves strongly suggested the presence of the pyrimidine ring in this molecule. The solution showed two maxima at 235 and 276 mμ under basic conditions, and only one at 242 mμ in acid (Fig. 2). The isolated pyrimidine fraction from thiamine has been shown to exhibit maxima at 235 and 277 mμ in basic solution and at 247 mμ in acid (19). The relative intensity of the extinction values of spot "D" were comparable to the values obtained with the known pyrimidine fraction prepared from thiamine. On the basis of these results, compound "D" appeared to contain the pyrimidine ring and an —S—S— bridge. This suggested a substance such as thiamine disulfide. However since the nitroprusside-cyanide reaction is not completely specific for —S—S— bridges, further proof for the presence of thiamine disulfide would still be desirable.

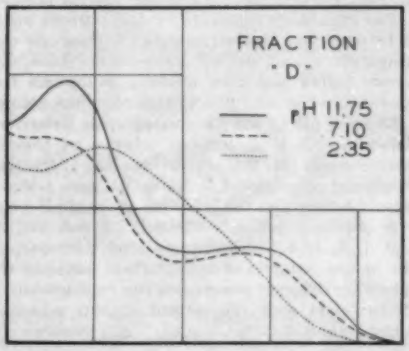

Fig. 2.—Absorption spectrum of fraction "D".

The conclusions from these experiments can be summarized as follows: there was no evidence of thiamine destruction in the acidic range, when the chromatographs were run immediately after the preparation of the solution. From pH 7 to 9, chromatography as well as the titration curve showed the transformation of thiamine to its carbinol form, and in addition the chromatogram indicated a partial transformation to thiochrome, especially at pH 8.5, and also to a small amount of a pyrimidine derivative (D). Above pH 9 a much larger fraction of the thiamine was destroyed. Under these conditions, the compounds present included the carbinol form of thiamine, thiochrome, a pyrimidine derivative that might be thiamine disulfide, another pyrimidine derivative and two unknown products.

Mode of Action of Dipotassium Phosphate.—The demonstration that K_2HPO_4 in the salt mixture can lead to a substantial loss of thiamine in diets for animal feeding (2) raised the question of how this degradation of thiamine compares with that due to NaOH. Hence chromatograms were run with 10% solutions of thiamine adjusted to pH 6.54 and 8.13 by means of K_2HPO_4. Thiamine solutions of the same concentration and adjusted to the same pH value with NaOH, were run on the same paper. The unused solutions were kept at 37° and aliquots taken for chromatography after 1, 2, 5, and 11 days.

In no case was any difference apparent between the degradation products obtained with K_2HPO_4 and those present after treatment with NaOH. This showed that at least qualitatively the effects of K_2HPO_4 were identical to those of NaOH at the same pH value.

Reaction Between Thiamine and Glucose.—Thiamine has been shown to react quite strongly in a Maillard type reaction when a dry mixture of the vitamin and glucose was heated at 85° (19). Thiamine as a free base was prepared by lyophilization of a 10% solution of thiamine hydrochloride brought to pH 6.54 with 9 *N* NaOH, and mixed with an equal amount of glucose. Ten per cent aqueous solutions were prepared with fresh mixtures or with mixtures that had been heated for 2 or 14 days. Fractions of 0.1 cc. of each solution were placed on chromatographic paper and run for fifteen hours with *n*-butanol-ethanol-water, 2/1/1, as a solvent. Six papers were run simultaneously so that various spray reagents could be used. The pool of spots obtained is represented on Fig. 3.

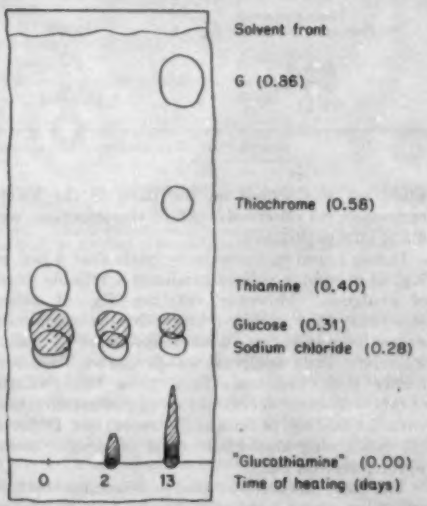

Fig. 3.—Chromatogram showing the reaction thiamine-glucose. Solvent system: *n*-butanol-ethanol-water, 2/1/1.

It appeared that the concentration of both thiamine and glucose was reduced considerably as heating continued. At the base of the paper, a new compound soluble in water and giving some tailing effect appeared. This compound possessed a very bright fluorescence. As demonstrated in the preceding section, thiamine destroyed by alkali yields pyrimidine derivatives characterized by very intense blue coloration when treated with a mixture of silver nitrate and bromphenol blue and washed. Paper chromatograms of the mixtures of glucose and thiamine were kept at room temperature for one month in order to allow thiamine to be progressively destroyed. After that period, the papers were tested with the silver nitrate-bromphenol blue reagent, washed and dried. All the spots of thiamine showed an intense blue coloration, a test which was also given in high intensity by the new lower spot. This suggested that a substantial fraction of the thiamine originally present was located in the new slowly moving spots. Secondary reactions were also involved in the process, at least two of which should be mentioned: oxidation of a minute amount of thiamine to thiochrome, and the formation of a new blue fluorescent product (R_f, 0.85).

SUMMARY

1. A paper chromatographic method was developed for separating certain degradation products of thiamine and the method applied to the mixtures that arise when solutions of the vitamin are brought to various pH's.

2. Freshly prepared basic solutions of thiamine contained the carbinol form of the vitamin, thiochrome, a pyrimidine derivative that appeared to be thiamine disulfide, another purine or pyrimidine derivative, and two unknown compounds.

3. Exposure to dipotassium phosphate resulted in the same products as those due to sodium hydroxide at comparable pH's.

4. Heating a dry mixture of thiamine and glucose resulted in a progressive destruction of these products and the formation of a new brown compound which showed a bright yellow fluorescence. The compound showed certain reactions of pyrimidine derivatives.

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The Antipyretic and Analgesic Activities of Thymotic Acid*

By J. E. O'BRIEN and R. K. THOMS

***Ortho*-and *para*-thymotic acids were shown to have greater analgesic and antipyretic activities than sodium salicylate. The LD_{50}'s in rats were also determined.**

THE STRUCTURAL RELATIONSHIP of *o*-thymotic acid to salicylic acid and thymol suggests that this compound might possess properties similar to those of its cognates. This acid was first prepared by Kolbe and Lautemann in 1860 (1). Spallino and Provenzal (2) in reporting on the preparation of several esters of *o*-thymotic acid stated that aqueous solutions of the acid and its sodium salt possessed marked antiseptic properties.

Experimental evidence supporting the report of these early investigations was presented by Street, Georgi, and Jannke (3) who, in a study of the phenol coefficients of *o*-thymotic acid and sixteen of its salts, found that several of the compounds possessed bactericidal and fungistatic properties.

Although this acid has been known for nearly a century, to the best of our knowledge no evaluation has been made of its pharmacological properties.

Para-thymotic acid was synthesized by Beltz and Jannke in 1956 (4). A literature search has revealed no studies of its pharmacological or antiseptic properties.

It is the purpose of this present work to determine if similarities exist between the pharmacological actions of the thymotates and the salicylates.

EXPERIMENTAL

The mono-sodium salts of *o*- and *p*-thymotic acids employed in this study were prepared by heating an appropriate amount of the acid with a chemically equivalent weight of sodium bicarbonate dissolved in water. The resulting solution was then diluted to a volume which contained the desired concentrations of the drug.

Determination of the Analgesic Activities.—The analgesic actions of the sodium salts of *o*- and *p*-thymotates were compared to those of morphine and sodium salicylate by a modification of a method devised by Smith, D'Amour, and D'Amour (5). Instead of a light focused on the rat's tail, a "Dolorimeter"[1] was used to apply a measured amount of heat. This consisted of a wire located in close proximity to a thermometer which could be heated electrically to various temperatures. A temperature was used which would cause the rats to withdraw their tails in five to six seconds. The time to respond was determined immediately before the injection of the drug to be tested and thirty minutes, one hour, and two hours after the injection. The scoring system which we used is given in Table I.

TABLE I.—SCORING SYSTEM FOR DEGREE OF ANALGESIA

Time Required to Produce a Response, Sec.	Points
7–7.9	1
8–8.9	3
9–9.9	6
10–11	10

Each rat was scored on the basis of the longest response time observed. All of the injections were made intraperitoneally.

It was found in preliminary trials that 4 mg. per Kg. of morphine sulfate produced a reliable degree of analgesia. However, 600 mg./Kg. of sodium salicylate were required to produce a comparable degree of analgesia. With lower doses of sodium salicylate, little analgesia was produced while doses higher than this were fatal to a large number of rats. These observations are in general agreement with the findings of Smith, D'Amour, and D'Amour (5) concerning this whole class of drugs (aminopyrine, aspirin, etc.)

The same method was used to determine the doses of sodium *o*- and *p*-thymotates which would produce the same degree of analgesia as 4 mg./Kg. of morphine sulfate and 500 mg./Kg. sodium salicylate. A summary of the results of the final experiments of this study is given in Table II. Each mean represents the observations on 30 animals.

The data in Table II show that the analgesia produced by the stated doses do not vary significantly. Thus, it may be stated that on a mg./Kg. basis sodium *o*-thymotate possesses 15 times the analgesic activity of sodium salicylate and $^1/_{10}$ that of morphine sulfate, while the *para* derivative possesses 1.9 times the activity of sodium salicylate and $^1/_{80}$ that of morphine sulfate.

Determination of Antipyretic Activities.—The antipyretic effects of the sodium salts of *o*- and *p*-thymotic acid compared to that of sodium salicylate

* Received May 3, 1957 from the School of Pharmacy, University of Connecticut, Storrs.

[1] Manufactured by Metro Industries, Long Island City, N. Y.

were determined by their ability to lower fever in in rabbits made hyperpyretic by the injection of attenuated *E. coli*.

TABLE II.—A COMPARISON OF THE ANALGESIA PRODUCED BY *ortho*- AND *para*-THYMOTATES WITH THAT OF MORPHINE AND SODIUM SALICYLATE

Drugs Compared	Mean Degree of Analgesia	S. E.[a]	t Value[b]
Morphine Sulfate, 4 mg./Kg.	5.1	±0.95	0.032
Sodium Salicylate, 600 mg./Kg.	5.06		
Morphine Sulfate, 4 mg./Kg.	5.1	±0.83	0.22
Sodium *o*-thymotate, 40 mg./Kg.			
Morphine Sulfate, 4 mg./Kg.	5.1	±0.85	0.16
Sodium *p*-thymotate, 325 mg./Kg.	4.96		
Sodium Salicylate, 600 mg./Kg.	5.06	±1.91	0.22
Sodium *o*-thymotate, 40 mg./Kg.			
Sodium Salicylate, 600 mg./Kg.	5.06	±1.03	0.09
Sodium *p*-thymotate, 325 mg./Kg.	4.96		

[a] *S. E.* is the standard error of the difference between the means and is calculated by the formula,

$$S.E. = \sqrt{s^2[(1/n_a) + (1/n_b)]}$$

where s is the standard deviation and n is the number of animals.

[b] The t value for 0.05 probability for 58° of freedom is 1.96. This is Fisher's t and is calculated by the formula, $t = d/S.E.$ in which d is the difference between the means.

The pyrogen employed in this investigation was made from fresh slants of *E. coli* incubated at 37.5° for forty-eight hours. A standard inoculum was prepared from these slants by placing one loopful of the culture in sufficient distilled water to give a suspension having a light transmittancy of 85% as determined in the Lumetron spectrophotometer. One-tenth ml. of this suspension was added to tubes containing 8 ml. of nutrient broth medium and incubated at 37.5° for forty-eight hours. Following incubation the cultures were attenuated by the addition of 0.2 ml. of 10% formalin to each tube. Fever was produced in the rabbits by the injection of 0.03 cc./Kg. of the attenuated culture into their marginal ear veins.

Observation of the temperature of the control rabbits over a period of six hours showed that the pyrogen maintained a constant temperature for at least this length of time.

The antipyretic activities of the thymotates were determined on the basis of the doses required to produce a decrease in temperature equivalent to that produced by 100 mg./Kg. of sodium salicylate. This evaluation was carried out in a series of four experiments employing a total of eight rabbits. The rabbits were rotated in such a manner that each rabbit was used once for each drug and once for a control. Each mean decrease in temperature given in Table III is the mean of eight observations.

TABLE III.—A COMPARISON OF THE ANTIPYRESIS PRODUCED BY *ortho*- AND *para*-THYMOTATES WITH THAT OF SODIUM SALICYLATE

Drugs Compared	Mean Decrease in temperature, °F.	S. E.[a]	t Value[b]
Sodium Salicylate, 100 mg./Kg.	3.39	±0.14	0.21
Sodium *o*-thymotate, 15 mg./Kg.	3.36		
Sodium Salicylate, 100 mg./Kg.	3.39	±0.10	0.16
Sodium *p*-thymotate, 60 mg./Kg.	3.27		

[a] This standard error is the same as that used in Table II.

[b] The t value for 0.05 probability for 6° of freedom is 2.45. The formula used to calculate these t values is the same as that used in Table II.

Acute Toxicity Studies.—The LD_{50}'s of sodium *o*- and *p*-thymotates were determined in albino rats. Each dose was given to a group of 10 rats (five males, five females) by intraperitoneal injection. The results were based on the number of rats dead in twenty-four hours. The statistical analysis of these results given in Table IV was done by the method of Wilcoxon and Litchfield (6).

TABLE IV.—THE ACUTE TOXICITY OF SODIUM *ortho*- AND *para*-THYMOTATE IN RATS

Drug	LD_{50}	Confidence Limit, LD_{50}	Slope	Confidence Limit, Slope
Sodium *o*-thymotate	178	165.2–191.6	1.20	0.98–1.48
Sodium *p*-thymotate	337	323.1–351.5	1.09	0.87–1.36

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Hypotensive and Toxicologic Properties of the Theophyllinate Salt of H-2 (N-Dimethylaminoethyl-4-Methyl-4, 7-Endoxyperhydro-Isoindole Dimethonium Chloride)*

By GISELA WINKLER,† W. EDWARD O'MALLEY, LEONARD M. RICE, and CHARLES F. GESCHICKTER

The theophyllinate[1] of H-2 has been studied for hypotensive properties. Mechanism of action studies indicated a synergistic effect between the ganglioplegic cation and the spasmolytic anion. Vasodepressive potency was significantly greater than that of the chloride salt.

IN A PREVIOUS COMMUNICATION from this laboratory, a methodology for comparing hypotensive agents was described (1). Also a new isoindole derivative, H-2 (N-dimethyl aminoethyl-4-methyl-4,7-endoxyperhydro-isoindole dimethonium chloride) was compared to hexamethonium chloride and to pentolinium bitartrate in regard to hypotensive properties. H-2 was synthesized by one of us (L. M. Rice) and its synthesis was reported elsewhere (2). The present report concerns a comparison of various salts of H-2 for hypotensive activities, and a study of some pharmacologic properties of one of these salts, the theophyllinate, which was selected for clinical appraisal.

METHODS

Blood Pressure Studies—The amate, monomucate, dimucate, chloride, nicotinate and theophyllinate of H-2 were compared for vasodepressive activity. Each salt was administered as a solution by stomach tube on the basis of equivalent quantities of active H-2 cation.

After the selection of H-2 theophyllinate as the most potent salt, further studies of its hypotensive and toxicologic properties were undertaken. The same trained groups of unanesthetized dogs (Groups I, II, and III) described in our former communication (1) were again employed for measurements of hypotensive responses. Each group is comprised of three dogs. Blood pressures were measured by femoral puncture and averaged at thirty minute intervals for two hours, then at four hours, six hours, nine hours, twelve hours, fifteen hours and eighteen hours after initial drug administration.

Eighteen-hour curves of blood pressure reponse to H-2 theophyllinate (Fig. 2) were plotted. Table I presents the average blood pressure together with the standard error at each time interval. These may be compared to those of H-2 chloride, reprinted for this purpose (Fig. 1). Both salts were administered at the following dosage levels, calculated on the basis of H-2 cation: 10 mg./Kg., 20 mg./Kg., and 50 mg./Kg.

Mechanism of Action Studies—Responses to test doses (1 cc. of 1:10,000 solution) of epinephrine hydrochloride were measured before and three hours after intramuscular administration of 25 mg./Kg. H-2 theophyllinate. Similar observations were made using doses of acetylcholine sufficient to elicit a 20 mm. Hg depression in blood pressure. On three nembutalized dogs (32 mg./Kg.), response of arterial blood pressure to rotation of the operating table to a 45° angle was tested and recorded by a mercury manometer. The carotid artery was canulated and the point of canulation kept at the same elevation throughout angular rotation. The same experiments were carried out with three dogs which were premedicated intramuscu-

TABLE I.—MEANS AND STANDARD ERRORS OF BLOOD PRESSURE

Time After H-2 T. Administration	Group I, 50 mg./Kg.	Group II, 20 mg./Kg.	Group III, 10 mg./Kg.
0 Min.	137 ± 4	129 ± 6	126 ± 5
30 Min.	115 ± 9	112 ± 1.7	112 ± 1.7
60 Min.	94 ± 4	79 ± 5	102 ± 8
90 Min.	89 ± 4	80 ± 1	102 ± 8
2 Hr.	88 ± 1.7	81 ± 1	102 ± 8
4 Hr.	88 ± 4	85 ± 1	108 ± 4
6 Hr.	96 ± 10	88 ± 1.7	114 ± 2
9 Hr.	92 ± 4	93 ± 1.7	117 ± 2
12 Hr.	103 ± 9	98 ± 4	122 ± 1.7
15 Hr.	107 ± 7	104 ± 6	127 ± 4
18 Hr.	105 ± 2	115 ± 8	127 ± 4

* Received August 8, 1957, from the School of Medicine and Dentistry, Georgetown University, Washington 7, D. C.
Supported by a research grant from the Geschickter Fund for Medical Research, Inc.
† Present address: Sloan-Kettering Institute for Cancer Research, New York 21, N. Y.
[1] This compound is presently available from Wyeth Laboratories, Inc., as Wy-1263.
Contribution from the Georgetown University Medical Center, Department of Pathology.

larly with 25 mg./Kg. of H-2 theophyllinate, two hours before operation.

The preganglionic and postganglionic fibers of the right superior cervical ganglion in the rabbit and the preganglionic fibers of the right vagus nerve in the cat were stimulated using a Harvard inductorium. Minimal stimuli for nictitating membrane contraction and bradycardia and vasodepression were determined. H-2 theophyllinate at 25 mg./Kg. dosage levels was administered intramuscularly. Twenty minutes later stimuli were repeated. Four rabbits and four cats were used.

Toxicity Studies.—Eight unanesthetized normal dogs were titrated at various dosage levels through an indwelling intravenous polyethylene catheter, according to a formerly described technique (1). H-2 theophyllinate was given as a 4% solution on the basis of cation. LD_{50}'s were estimated by the method of Litchfield and Wilcoxon (3). White male rats of the Wistar strain, 180 to 220 Gm. were used. Chronic experiments on rats were undertaken at three dosage levels: 0.5 mg./rat/day, 5.0 mg./rat/day, 10.0 mg./rat/day by intramuscular administration. Ten rats were employed at each dosage level. An isotonic sodium chloride control group was also included. Growth curves were plotted and all animals were sacrificed at the end of six months. Liver, kidneys, adrenals, brain, lungs, bone marrow, and intestines were macroscopically and microscopically examined. Determinations were also carried out for blood levels of sugar, urea nitrogen, bilirubin, creatin, creatinine, uric acid, and for albumin to globulin levels, hematocrit, WBC, and differential.

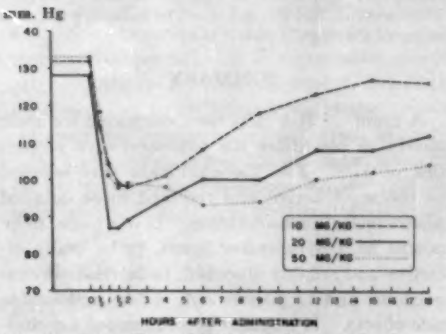

Fig. 1.—The effect of H-2 chloride on blood pressures of unanesthetized dogs.

RESULTS

The vasodepressive activity of H-2 chloride was not potentiated by substitution of the amate, nicotinate, monomucate or dimucate salts. The theophyllinate was significantly synergistic to the ganglionic blocking action of H-2. This fact led to its selection as the particular salt for further pharmacologic studies.[2]

Blood Pressure.—Eighteen-hour readings are presented graphically according to methods previously published (1). Figure 1 illustrates the effect of 50, 20, and 10 mg./Kg. of H-2 cation when administered as chloride. The formerly selected and trained "blood pressure" dogs of Groups I, II, and III were employed respectively. Figure 2 illustrates the effect of 50, 20, and 10 mg./Kg. of H-2 cation when administered as the theophyllinate salt. Groups I, II, and III dogs were used again.

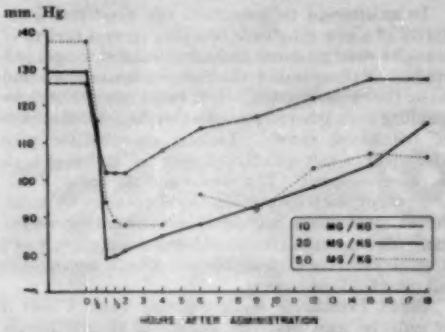

Fig. 2.—The effect of H-2 theophylline on blood pressures of unanesthetized dogs.

Studies on Mechanism of Action.—The following experiments appeared to demonstrate ganglionic blocking activity as the chief mechanism of action of H-2 theophyllinate. Injections of test doses of epinephrine and acetylcholine were fully effective after administration of H-2 theophyllinate to the dog. Postganglionic but not preganglionic excitation of the superior cervical sympathetic ganglion elicited a pupillary dilatation in the rabbit. Rotating an anesthetized dog to an angle of 45° produced a 5 to 20 mm. Hg depression in blood pressure. If the animal was previously medicated with 25 mg./Kg. of H-2 theophyllinate, a depression of 35 to 40 mm. Hg resulted in addition to the vasodepression normally elicited. In the anesthetized cat, bradycardia and vasodepression, resulting from mild right vagal stimulation, were abolished. Also the pressor effects of nicotine were no longer elicited after administration of H-2 theophyllinate. Evidence of both decreased sympathetic and decreased parasympathetic impulses to the eyes followed administration of H-2 theophyllinate to intact dogs. This was manifested by dilated pupils and relaxed nictitating membrane in the presence of enophthalmia.

Toxicity Studies.—Of the eight unanesthetized dogs, titrated with a 4% H-2 theophyllinate solution, four died at a dosage level of 100 mg./Kg. The four remaining were titrated at a dosage level of 80 mg./Kg.; they survived. It was therefore estimated that the approximate median lethal dose was 90 mg./Kg. in the dog on a six minute titration basis. The twenty-four hour intraperitoneal LD_{50} on male Wistar rats of 180 to 220 Gm. was estimated

[2] The synthesis of the theophyllinate salt for synergism was suggested by Dr. John C. Krantz, Jr., Department of Pharmacology, University of Maryland.

as 145.0 mg./Kg. with 95% confidence limits of 126.0 mg./Kg. to 164.0 mg./Kg. Chronic experiments over a six-month period (0.5, 5.0, and 10.0 mg./rat/day) were found to produce no specific changes attributable to the compound, in the macroscopically and microscopically examined organs. No significant changes were observed in the blood chemistry or in the growth curves.

DISCUSSION

In an attempt to potentiate the hypotensive activity of a new ganglionic blocking agent a variety of its salts were prepared and administered to test animals. Of those tested, the theophyllinate was found to be the most effective. It is well known that theophylline has a spasmolytic effect on the smooth muscle of the blood vessels. Linking theophylline with H-2 yields a salt which combines the action of both in one compound. The cation and the anion of this compound, each depressing blood pressure by a different mechanism, are therefore mutually synergistic. This permits the use of lower dosage levels and thus reduces the undesirable side effects, common to all ganglioplegic agents.

Blood Pressure.—By comparing Figs. 1 and 2 it may be seen that the linkage of theophyllinate to H-2 significantly potentiates the vasodepressive activity. A significant response in blood pressure was considered to be a depression of 25 mm. Hg or more.

Although this response occurred only briefly at a dosage level of 10.0 mg./Kg. of H-2 theophyllinate the blood pressure did not return to its original level for a period of fifteen hours. With 20 mg./Kg. significant response occurred after one-half hour and persisted for a thirteen-hour period, not returning to original levels even after eighteen hours. With 50 mg./Kg. significant response was achieved after one-half hour and persisted. A measurement made twenty-six hours after administration of the 50 mg./Kg. dosage level indicated an average pressure of 115.

The usual ocular manifestations were present at all dosage levels: dilatation of pupils and loss of pupillary reflex, relaxation of the nictitating membrane, injection of the conjunctiva, and widening of the palpebral fissure. However all dogs appeared to be in healthy condition and were able to micturate and defecate regularly and did not suffer apparent dryness of the oral mucous membranes. Sedation usually observed with ganglionic blocking agents did not occur. On the contrary, the dogs seemed to be active and alert, perhaps owing to central stimulating properties of theophylline.

Toxicity Studies.—The intravenous median lethal dose of H-2 theophyllinate has been approximated at 90 mg./Kg. in contrast to the previously recorded estimations (1) of hexamethonium and pentolimium at 35 mg./Kg. and 10 mg./Kg., respectively. Considering the fact that H-2 chloride has an exceedingly low median lethal dose, approximated at 200 mg./Kg., one must conclude that the addition of theophylline has somewhat narrowed the range of safety. Nevertheless, H-2 theophyllinate, with the estimated intravenous median lethal dose of 90 mg./Kg., is still relatively innocuous.

The twenty-four hour LD_{50} on rats of 145.0 mg./Kg. also indicates a relative lack of toxicity. On the basis of the foregoing studies, this agent is recommended for clinical appraisal.

CONCLUSIONS

From a group of H-2 salts prepared and studied, the theophyllinate was selected for its ability to potentiate the hypotensive activity of the basic compound.

The fundamental requirements for a useful hypotensive agent, appear to be fulfilled by H-2 theophyllinate. It is orally effective at all the dosage levels tested. At 20 mg./Kg. its activity persists longer than fifteen hours. At 50 mg./Kg. the duration of its activity exceeds twenty-six hours. Its margin of safety appears adequate. The usual side effects due to its action as a ganglionic blocking agent were present, but did not seem to influence the well being of the dogs.

SUMMARY

A group of H-2 salts were compared for their ability to potentiate the vasodepressive properties of H-2. The theophyllinate was selected as the most active and received more detailed pharmacologic consideration. It was found to be potent as a hypotensive agent, to be orally effective and reliably absorbed, to be relatively innocuous, and to possess only a few undesirable side effects. It has been recommended for clinical evaluation.

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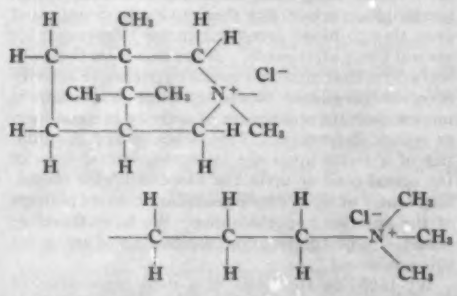
Studies on the Antihypertensive Action of N-Dimethylaminopropyl-1-Methyl-8, 8-Dimethyl-3-Azobicyclo (3,2,1) Octane Dimethochloride (Wy-1395)*

By W. EDWARD O'MALLEY, GISELA WINKLER HAEMMERLI, LEONARD M. RICE, and CHARLES F. GESCHICKTER

Compound Wy-1395[1] has been tested for hypotensive potency and toxicity in both intact and anesthetized preparations. This agent was extremely potent in hypotensive properties at a low dosage level and appeared to possess both central depressive and ganglioplegic properties.

THE VASODEPRESSOR ACTIVITY of the unsymmetrical bis-methonium compound, N-dimethylaminoethyl-4-methyl-4,7-endoxyperhydroisoindole dimethonium chloride also labeled H-2 and Wy-1263 has previously been reported (1). The hypotensive activity of this compound was surprising in light of structure *versus* activity studies of Paton and Zaimis (2). These investigators had found symmetrical bis-methoniums to possess greatest potency when the methylene chain had five or six carbons. Apparently this observation holds true only for symmetrical compounds, for H-2 possessed greater activity than its three-carbon analog, and activity diminished to negligible levels as the methylene chain was lengthened beyond three carbons.

As an extension of our studies on unsymmetrical bis-methoniums, the compound Wy-1395 has been reported (3, 4), and its structure is shown below.



It is the purpose of this communication to present the hypotensive and toxicologic properties of this compound. Since these studies have been conducted in accord with the methodology presented in our first report (1), reference to the latter will enable potency and toxicologic comparison to the agent H-2 as well as to pentolinium and hexamethonium. The potency of these three compounds has been expressed in graphical form in our first report; the potency of Wy-1395 is similarly expressed in this report (Fig. 1). Acute toxicity studies were performed on Wy-1395 in the same fashion as the corresponding studies on H-2, pentolinium and hexamethonium.

METHODS

Blood Pressure Measurements.—The blood pressures were recorded by direct femoral arterial puncture in the manner previously described (1). Three groups of dogs were used. Group I received 5.0 mg./Kg., group II received 2.0 mg./Kg., and group III received 1.0 mg./Kg. Each group was comprised of three dogs. Dosage of compounds was calculated on active cation basis. The drug was administered in aqueous solution via stomach tube. An initial blood pressure determination was made just prior to drug administration. Other determinations were made at thirty minute intervals for two hours, and then at four, six, nine, twelve, fifteen, eighteen, and thirty-six hours after the time of administration.

Acute Toxicity.—Compound Wy-1395 was infused intravenously into five dogs at a uniform rate over a six minute time period. The compound was administered as a 1.0% solution of cation. Unanesthetized animals were used by employing the technique previously described (1). Twenty milligrams per kilogram was administered to all five animals.

Twenty-four hour intraperitoneal LD_{50}'s were determined on 180- to 220-Gm. male Wistar rats by the method of Litchfield and Wilcoxon (5). Seven groups of 10 rats each were injected with graded doses of 1.0% Wy-1395.

Chronic Toxicity.—Three groups of 15 white male Wistar rats were injected daily intramuscularly with 1.0 mg./Kg., 2.0 mg./Kg., and 5.0 mg./Kg. of Wy-1395. A fourth group of 15 rats served as a control group. All animals were weighed biweekly and sacrificed at the end of three months.

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Supported by a research grant from the Geschickter Fund for Medical Research, Inc.
The technical assistance of Betty E. Collette is gratefully acknowledged.

[1] This compound was obtained from Wyeth Laboratories, Inc., for experimental and clinical investigation.

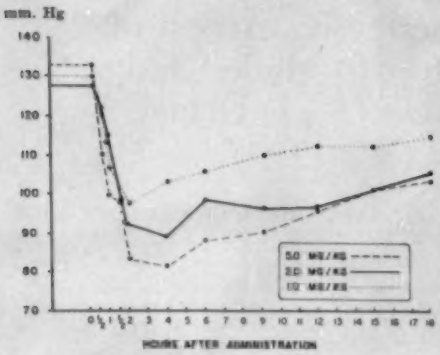

Fig. 1.—Effect of Wy-1395 on blood pressure of unanesthetized dogs.

TABLE I.—MEANS AND STANDARD ERRORS OF BLOOD PRESSURE

Time After Wy-1395 Administration	Group I 5.0 mg./Kg.	Group II 2.0 mg./Kg.	Group III 1.0 mg./Kg.
0 Min.	133 ± 3	127 ± 4	130 ± 3
30 Min.	110 ± 4	122 ± 3	120 ± 3
60 Min.	100 ± 8	115 ± 5	113 ± 6
90 Min.	98 ± 6	99 ± 3	106 ± 5
2 Hr.	84 ± 7	92 ± 7	98 ± 4
4 Hr.	83 ± 4	90 ± 9	103 ± 6
6 Hr.	88 ± 3	100 ± 8	106 ± 4
9 Hr.	91 ± 5	98 ± 3	111 ± 6
12 Hr.	97 ± 9	98 ± 4	113 ± 6
15 Hr.	103 ± 4	103 ± 7	113 ± 3
18 Hr.	107 ± 4	105 ± 5	116 ± 3
36 Hr.	110 ± 4	106 ± 6	111 ± 6

RESULTS

Blood Pressure Measurements.—Following previous procedure a curve has been plotted for each dosage group. The pressure determinations made at each time interval have been averaged from the readings of the three dogs comprising a group. This serves to smooth the response curve. The curves for all groups are presented in Fig. 1. The mean pressure reading and standard errors are expressed in Table I.

Acute Toxicity.—All five dogs administered 20 mg./Kg. of Wy-1395 via intravenous infusion demonstrated accentuated symptoms of ganglionic blockade, particularly ophthalmic symptoms. Pupils were widely dilated and photophobic, and the conjunctivae were deeply injected. Perhaps owing to postural hypotension, these animals were unable to remain standing for more than a few seconds. They displayed a general lack of somatic activity for several hours after drug treatment. One animal expired five minutes after the termination of the titration owing to respiratory depression. The other four animals showed some respiratory depression during intravenous titration, but this effect subsided within a few minutes after titration.

The twenty-four hour intraperitoneal LD_{50} on Wistar rats of approximately 200 Gm. body weight was 138 mg./Kg. with 95% confidence limits of 124 to 152 mg./Kg.

Chronic Toxicity.—No depression in growth curves was noted at any of the dosage levels employed. Gross and microscopic post-mortem examination revealed no lesions attributable to the agent administered.

DISCUSSION

Reference to Fig. 1 indicates that Wy-1395 has very effective hypotensive properties at the oral dosage level of 2.0 mg./Kg.; and it appears that 5.0 mg./Kg. is an excessive dosage level. These data indicate that Wy-1395 has hypotensive potency in excess of any quaternized ganglioplegic agents presently in wide use. Its interim of activity is most prolonged, significant vasodepression existing thirty-six hours after drug administration. The acute toxicity studies indicate a wide margin between the dosage level necessary to produce a hypotensive response and the dosage level to elicit death in the dog. It was observed during the course of this study that the symptoms of ganglionic blockade, which usually accompany the hypotensive effects of ganglioplegic agents, occasionally did not obtain with the agent Wy-1395. This was unexpected because preliminary mechanism of action studies had indicated complete blockade of the superior sympathetic ganglion to transmission of impulses upon stimulation of preganglionic fibers, as indicated by lack of pupillary constriction. These tests were conducted in rabbits using an intravenous route of administration. However, a stereotyped set of symptoms almost always accompanies administration of quaternary ganglioplegic agents in the dog. These include endophthalmia, pupillary dilatation, relaxation of the nictitating membrane, conjunctival injection, dryness of the snout, lack of salivary secretions and dryness of the foot pads, and at high dosage levels, urinary and fecal retention and tachycardia. The occasional lack of these by-effects was suspicious. Further observation revealed that very frequently these foregoing symptoms were present after initial drug administration indicating initial ganglioplegic action, but then gradually disappeared even though blood pressure remained depressed for several hours afterwards. It appears from these observations that although initial hypotensive activity involves ganglionic blockade, there is perhaps a more important component of activity in some form of centric depression. This indicates the possibilities of activity upon the intermediolateral horn of the spinal cord or upon the vasoconstrictor medullary center or upon supra-medullary centers perhaps of the anterior hypothalamus. We have therefore been led to postulate a dual mechanism of action for this compound.

Wy-1395, on the basis of a dual mechanism of action and a relatively low toxicity appears to merit clinical trial in the hypertensive vascular diseases. Clinical studies have therefore been initiated.

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Some New Constituents of Mint Oils*

By ROBERT H. REITSEMA†

New constituents of the oil of several species of mints have been identified. Menthofuran is found to be a major constituent of *M. aquatica*. Diosphenol, piperitenone oxide, diosphenolene, piperitone, piperitenone, limonene, and cineol have been identified in *M. sylvestris*. Jasmone has been isolated from American spearmint oil. Dihydrocarvone has been found in several species of carvone containing mints.

SEVERAL of the more interesting essential oils of the mint species previously have not been well characterized chemically. In certain cases there was no indication in the literature of the major compounds present. As a result of extensive use of these species in a hybridization program, it was desired to fill in some of the missing information.

A study of the spearmint type oils has revealed the presence of dihydrocarvone as a constant companion to carvone although the ratios of the two materials differ. Confirmation of this by preparation of the dihydrocarvone derivative from the whole oil was convenient for *Mentha niliaca* where the amount of dihydrocarvone is appreciable. In the case of a spearmint type oil such as American or Scotch spearmint where there is a very great amount of carvone present, it was preferable to remove most of the carvone by extraction with neutral bisulfite prior to treatment with dinitrophenylhydrazine.

A more unusual type of compound has been isolated from American spearmint oil. Jasmone was found in a fraction of the oil after removal of carvone and alcohols. The occurrence of this material in peppermint oil has been reported (1) and thus it is a reasonable material to find in another mint species. It does not appear that jasmone is closely connected with the terpene syntheses. It is quite possible that it is not produced in the oil glands, but is a product of other parts of the plant similarly to the leaf alcohols.

M. aquatica gives an unusual oil, and there was no indication given in previous work of the compounds which were present in it. It was observed that it gave a heavy blue spot near the lead edge of the solvent when chromatographed by the previously described technique (2, 3). The whole oil had a positive rotation. The infrared absorption of the whole oil showed the presence of bands corresponding to those of menthofuran, a compound well known in peppermint oil. These observations suggested the presence of considerable amounts of menthofuran. A standard infrared menthofuran assay showed the presence of at least 40 per cent methofuran. This was confirmed by the actual isolation of very pure menthofuran in an overall yield of 34 per cent. The adduct with maleic anhydride also corresponded in all respects to authentic adduct prepared from menthofuran. This extremely high concentration of menthofuran is of considerable interest since under some cultural conditions American peppermint oil is obtained with a menthofuran content of 15 per cent or more instead of the usual two to five per cent. A high menthofuran content is not considered desirable from a flavor standpoint, and it seems that further study of *M. aquatica* may give some information about the mechanism of menthofuran formation in mint plants.

M. sylvestris gives an oil which has already been shown to contain large amounts of piperitone oxide (4). It now has been possible to determine the nature of some of the other materials present. The most interesting new material found in the oil was diosphenol. This is the first oil of the mint species found to contain diosphenol although the compound is well known as a constituent of various species of *Barosma* (buchu leaves) (5). It also is a constituent of some oils of the *Lippia* genus on the basis of interpretation of published work (4, 6). Since diosphenol is readily obtained from the piperitone oxide it is a reasonable compound to be found along with the oxide.

Chromatography and fractional distillation showed the presence of small amounts of piperitone oxide and diosphenolene. These compounds were previously found to be major constituents of *M. rotundifolia* (7). Piperitone and piperitenone themselves also were found in the oil. A brief study of the hydrocarbons in the oil showed that limonene and cineol were present in appreciable amounts.

* Received June 13, 1957, from the Chemical Laboratory of the A. M. Todd Company, Kalamazoo, Mich.

† Present address: The Ohio Oil Company, Denver Research Center, Littleton, Colo.

The author wishes to acknowledge the technical assistance of V. J. Varnis and W. E. Faas, the helpful comments of M. I. Murray, chief geneticist, A. M. Todd Co., and the encouragement of this work by W. A. Todd.

M. rotundifolia was studied further and found to contain small amounts of piperitone oxide and diosphenol.

EXPERIMENTAL[1]

The methods of distillation and chromatography of the oils have been described previously (2, 3, 8). The extraction of the spot on the chromatogram was a new approach and gave a liquid solution which could be inspected for ultraviolet absorption of the particular compound.

Dihydrocarvone in Spearmint Type Oils.—Identification of dihydrocarvone in a sample of oil of *M. niliaca* was by addition of 1.0 ml. of a saturated ethanolic solution of 2,4-dinitrophenylhydrazine to 0.5 Gm of the oil in 5 ml. of ethanol. The carvone derivative which appeared very quickly was separated by filtration. Addition of water in portions to the filtrate gave a precipitate which yielded dihydrocarvone dinitrophenylhydrazone, m. p. 142–144° after crystallization from ethanol. This melted without depression when mixed with authentic derivative.

Similarly, from American spearmint or *M. crispa* oil which had been freed of carvone through bisulfite extraction and of alcohols by distillation from boric acid was isolated the same dinitrophenylhydrazone m. p. 143–144°. The semicarbazone also prepared from this fraction melted at 186–189°. Neither derivative showed a melting point depression when mixed with authentic material.

Jasmone in Spearmint Oil.—Distillation of the noncarvone, nonalcohol portion of American spearmint oil through a ten-theoretical plate column gave a fraction boiling 77–81° (2 mm.), α_D −27.25°, n_D^{20} 1.4948. Chromatography indicated the presence of an unusual ketone. The semicarbazone melted at 200–201° as for the jasmone derivative. Preparation of the dinitrophenylhydrazone derivative confirmed the presence of jasmone by its melting point, 120–120.5° (reported 121–122.5°(9)) and by its elemental analysis.

Anal.—Calcd. for $C_{17}H_{20}N_4O_4$: C, 59.29; H, 5.85; N, 16.27. Found: C, 59.56; H, 5.65; N, 16.33.

Methofuran in M. aquatica Oil. The adduct from 7.87 Gm. of *M. aquatica*, (d_{25}^{25} 0.947, n_D^{20} 1.4798, $[\alpha]_D$ 46.5°) and 2.0 Gm. of maleic anhydride was decomposed to give 34.5% of the original oil as methofuran, n_D^{20} 1.4857, $[\alpha]_D^{20}$ 94.6° (reported (10) n_D^{25} 1.4832, $[\alpha]_D^{25}$ 92.5°). The maleic anhydride adduct recrystallized from benzene melted at 131–132° (132–133° reported (10)) and gave no melting point depression when mixed with authentic material.

Infrared analysis of the whole oil of *M. aquatica* indicated the presence of 40.0% methofuran by absorption measurements at 733 cm.$^{-1}$ in a 0.05-cm. cell. This absorption point is one of those mentioned by Naves (11).

Piperitenone Oxide (1,2-Epoxypulegone) in M. sylvestris Oil.—Redistillation of fractions of *M. sylvestris* oil boiling above 79° at 3 mm. through a 24-inch Podbielniak Heli-Grid column gave a fraction boiling at 99° (5 mm.) n_D^{20} 1.5004, α_D^{25} −17.16°. Stirring 1.0 Gm. of this fraction with 0.6 Gm. of sodium acetate and 0.9 Gm. of semicarbazide hydrochloride for three hours gave 0.53 Gm. of a white solid, m. p. 180–181°, which was crystallized from methanol giving a product melting at 178–179° which did not depress the melting point of authentic piperitenone oxide. The ultraviolet absorption showed a maximum at 274.5 mμ (reported for piperitenone oxide semicarbazone (7) m. p. 177.5–178°, $\lambda_{Max.}^{EtOH}$ 274.5 mμ).

Piperitone in M. sylvestris Oil.—Redistillation of *M. sylvestris* fractions boiling above 79° (3 mm.) gave a fraction boiling at 92–96° (8 mm.) n_D^{20} 1.4635. Chromatography showed a ketone present at the same location as piperitone. Extraction of a corresponding area from an unsprayed chromatogram gave a solution in ethanol with an ultraviolet absorption maximum at 235 mμ as found for piperitone. The semicarbazone melting at 226–227° (reported for piperitone semicarbazone 226–227° (12)) showed no mixed melting point depression when mixed with authentic material.

Piperitenone in M. sylvestris Oil.—From the same chromatogram used for the above identification of piperitone a spot at the piperitenone area was extracted. This showed a maximum ultraviolet absorption at 245 mμ as for authentic piperitenone. The semicarbazone isolated in poor yield melted at 243–244° and gave no depression in melting point when mixed with piperitenone semicarbazone.

Limonene in M. sylvestris Oil.—Preparation of the tetrabromide from the *M. sylvestris* fraction boiling 95–96° (53 mm.), n_D^{20} 1.4706, in amyl alcohol-ethyl ether gave after crystallization from ethyl acetate white crystals, m. p. 103–104°. The melting point was not depressed when mixed with authentic limonene tetrabromide. Chromatography of the fraction also indicated the presence of limonene.

Cineol in M. sylvestris Oil.—The fraction of *M. sylvestris* oil, b. p. 94–95° (53 mm.), gave with a 50% aqueous solution of resorcinol a copious white precipitate, m. p. 83–85°, which gave no depression of melting point when mixed with authentic cineol-resorcinol complex (reported m. p. 80–85° (13)). The complex with phosphoric acid was also prepared readily. The fraction gave a dark spot on a chromatoplate on heating which was indistinguishable from that produced by authentic cineol.

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[1] The mint species used as the source of the oils in this work were grown under the direction of M. J. Murray of this laboratory and herbarium specimens have been deposited at the Cornell University Herbarium and at the Missouri Botanical Gardens, St. Louis.

A Biogenetic Arrangement of Mint Species*

By ROBERT H. REITSEMA†

On the basis of various chemical and genetic studies a scheme of biosynthesis of the compounds found in the mint oil from available species is presented. The several most interesting species also are classified as either peppermint, spearmint, or acyclic types. A scheme of conversion of the individual ketones within each type is given and an arrangement of the mint species themselves on this basis is indicated.

COMBINATION of the various studies which have been made on mint species now permits the proposal of a biosynthetic scheme which can account for the variations between the species. Study of the chemical composition of the species (1), comparison of oil from young and older leaves taken from the same plant (2), assays on commercial crops during the growing season, and observations during genetic work with mints (3) have all led to a common explanation. Contrary to older views, the biochemistry of these essential oils apparently is not complex and only a few different types of reactions are involved. Predominantly only cyclization, reduction, and a special type of oxidation appear to be necessary for explaining the formation of the different constituents of the oils.

Mint oils have been grouped into the spearmints, the peppermints, and the lemon mints (4, 5). This is on the basis of the presence of 2-oxygenated-*p*-menthanes, 3-oxygenated-*p*-menthanes, and acyclic compounds, respectively. These three groups are distinct and no evidence has been obtained in the present program for the coexistence of both 2- and 3-oxygenated-*p*-menthanes in the oil from a single mint plant. The suggestions in the literature of possible coexistence are not in each case supported by adequate chemical identification. Furthermore, until the development of techniques for inspection of oils from a single plant (2), combined with the controlled cultivation of pure species, the chance of investigating oils from mixed species or from non-homozygous strains was always present. This is a significant source of trouble since many of the mint species have similar botanical appearance and since there has always existed a great confusion regarding the nomenclature of the mint species.

The formation of the constituents of oils of the mint species can be explained best from the point of view that the activity of enzyme systems differ among the species. The overall scheme is summarized in Fig. 1. The cyclization reaction is not promoted in oil of *M. citrata* and, therefore, it contains acyclics like citral and linalool. The presence of the enzyme for cyclization gives a cyclic intermediate which previously was shown to be converted to either a peppermint or a spearmint type oil by the change in a single gene. The spearmint and peppermint series oils are extremely similar in the type of compounds present. Except for the location of the oxygen group on the ring, corresponding compounds are found in each series. Once one or the other of these types is formed from the intermediate, the further reactions can be very similar.

The spearmint oils contain more unsaturated compounds and much less saturated alcohols. Thus far, no odd side reaction products have been observed in oils from species of the spearmint type. The spearmint oils appear to be simply formed by the production of carvone and reduction of carvone to a greater or lesser extent depending upon the species to dihydrocarvone and carvomenthone as suggested previously (4). Still lesser amounts of the corresponding alcohols are found.

The peppermint type oil compounds also arise from a reduction scheme of synthesis similar to the spearmints. Piperitenone would be the compound analogous to carvone. However, the reduction process as shown in Fig. 1 is much more prominent than in the spearmint oils and there is little piperitenone in peppermint oil itself. Menthol, the completely saturated alcohol, is the major product of the normal synthesis. The interesting feature of certain peppermint type oils is that there appear to be steps in the reduction which do not occur rapidly or at all and then an epoxidation reaction takes over. This is shown in *M. rotundifolia* where compounds like piperitenone oxide and diosphenolene (6) can be isolated and apparently piperitenone is not reduced by the normal peppermint type process. Similarly, *M. sylvestris* compounds like piperitone oxide and diosphenol (7) could be produced from either the *M. rotundifolia* compounds or by epoxidation of piperitone. Failure of pulegone reduction to occur would give a species such as *M. pulegium* with its very high pulegone content. Menthofuran may arise by an oxidative process on pulegone leading to *M. aquatica*.

The compounds present in the most important of the peppermint type oils studied are summarized in Table I. Here are given the per cent composition of the average or typical oils of the species where that information is available. The emphasis is upon the ketones since they have been the most readily detected and apparently are reliable indicators of the alcohols present in the same oil. The progression of increasingly more reduced compounds as one goes along the table is one of the evidences for the arrangement of the species as presented. It is of interest to note that, although the formation of one or the other of the three types is exclusive, the reactions within the peppermint type oils, for instance, is not exclusive and reductions to the next stage may occur to different degrees.

The place of terpene hydrocarbons in the scheme of the oil synthesis is apparently of less significance for species differentiation. These compounds could arise in several ways, such as by elimination of a proton from a cyclic intermediate and subsequent

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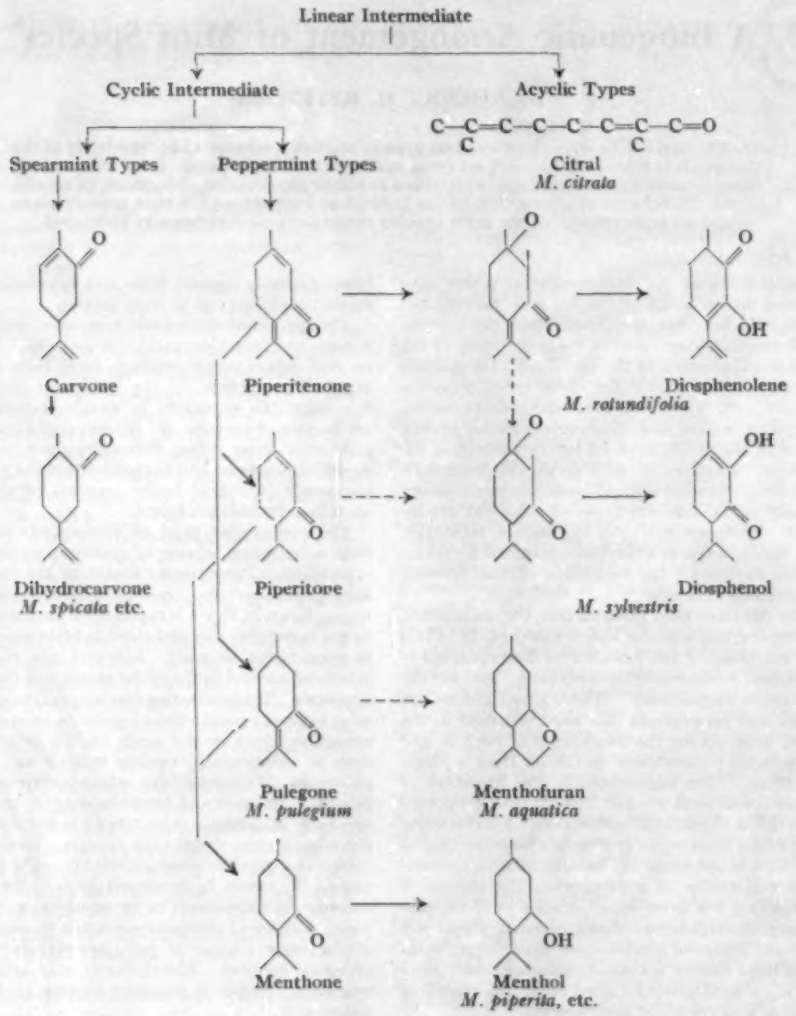

Figure 1.

TABLE I.—RELATIVE AMOUNTS OF CONSTITUENTS IN PEPPERMINT TYPE OILS[a]

	M. rotundifolia	*M. sylvestris*	*M. pulegium*	*M. aquatica*	*M. piperita*	*M. arvensis* var. *piperascens*
Piperitenone	..	x	..	..	1%	x
Piperitenone oxide	50%	x	..	..	..	..
Diosphenolene	x	x[b]	..	..	..	..
Piperitone	x[b]	..	3%	x[b]	1%	x
Piperitone Oxide	5%	45%	..	..	..	..
Diosphenol	x[b]	3%	..	..	..	..
Pulegone	..	..	80%	x[b]	2%	x
Menthofuran	..	..	..	40%	2–15%	0%
Menthone	..	..	4%	x[b]	25%	5%
Menthol	..	..	5%	..	50%	80%

[a] Absence of quantitative data indicates lack of data rather than an implied absence of the compound in the oil.
[b] Identified by chromatography and ultraviolet absorption.
x Indicates presence without quantitative data.

isomerization along paths suggested by Ruzicka (8). The addition of oxygen to a terpene such as limonene to produce carvone is a known autoxidation reaction which should not be ignored, but it does not explain the absence of carvone in all but a few limonene containing oils. It appears that the proposal of a hydrocarbon as an intermediate compound in the biosynthesis of the oxygenated compounds is unnecessary.

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Antiveratrinic Activity of Lactones and Peroxides*

By DUANE G. WENZEL and CARROLL M. SMITH

Several saturated and unsaturated γ-lactones and peroxides were tested for their antiveratrinic activity on the frog sartorius muscle. Some of the unsaturated lactones and a hydroperoxide were active. This action resembled the antiveratrinic effect of quinine more closely than that of ouabain. The activity of the unsaturated lactones could not be satisfactorily related to potential peroxide formation.

CARDIAC GLYCOSIDES and certain veratrum alkaloids share a number of pharmacological activities and yet are antagonistic in one respect. Each has a positive inotropic action and similar effects on the rate of impulse production and on the rate of conduction (1). For example, the alkaloids cevine and veratridine cause a typical digitalis-like systolic standstill of the isolated frog heart (2) and improve the isolated failing heart (3).

The veratrinic response, a prolonged contraction of skeletal muscle following the administration of certain veratrum alkaloids and a single electrical stimulation, is not shared by the cardiac glycosides. The latter are among the most potent inhibitors of this response (4). As the erythrophleum alkaloids have parallel antiveratrinic and positive inotropic activities, it would appear that these actions are mechanistically related (5).

An unsaturated lactone group is not only essential to the activity of the cardiac glycosides but the separate lactones induce a positive inotropic action on the frog heart. This activity has been related to their ability to form peroxides (6). As these lactones are not active on the mammalian heart (7, 8) it has not been possible to conclude that peroxide formation is related to the functioning of the cardiac glycosides. That the glycosides may be active through their peroxides is indicated by the work of Procter (9) who found that digitoxin in solution takes up oxygen and that this oxygen-complex is more effective on digitalis-sensitive enzyme systems than is digitoxin alone.

The present study was undertaken to determine if simple lactones inhibit the veratridine response and if this inhibition parallels their ability to form peroxides.

EXPERIMENTAL

Materials.—The compounds used in this study and their sources are listed in Table I. *Gamma*-ethyl-3,4-angelica lactone was made according to the method devised by Haynes and Jones (10, 11) for the preparation of γ-methyl-α,β-angelica lactone; 3-methyl-1-pentyn-3-ol was used instead of 3-methyl-1-butyn-3-ol.

All lactones tested were checked for identity on the basis of boiling point, refractive index, and infrared absorption spectra. Carbonyl and carbon-hydrogen stretching peaks were obtained with all the lactones. A peak at 11.8 microns attributable to peroxide vibrations was obtained with tertiary butyl hydroperoxide, but peroxide peaks were not demonstrable with the lactones in the chloroform solution as used for infrared analysis. Other workers have demonstrated in polarographic studies that the unsaturated lactones form peroxides in a slightly basic aqueous medium (12, 13).

Method.—The general procedure including solutions and apparatus used to determine the veratrinic response was essentially that of Arora (4). Both

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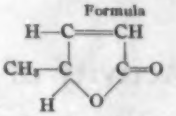
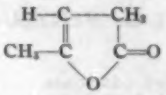
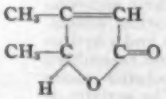
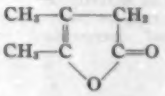
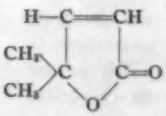
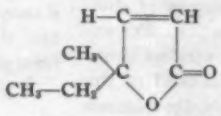
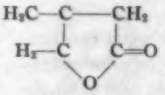
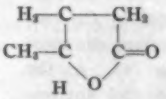
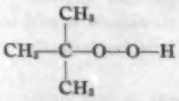
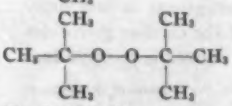
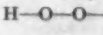
sartorius muscles of a female frog, *Rana pipiens*, were placed in twin 10 ml. baths and connected to gravity levers for simultaneous recording on a single drum. The muscles were stimulated through a platinum electrode in the base of each bath and a platinum hook in the top of the muscle.

The veratridine response was obtained by adding veratridine, 1:10,000,000 final concentration, and stimulating for 0.5 milliseconds with a 40 volt square wave stimulus. This duration and amplitude was adequate except when the muscles were weak or fatigued. Optimum bath temperature was 23–25°.

TABLE I.—ANTIVERATRINIC ACTIVITY

Compound	Source	Formula	Liminal Initial Antiveratrinic Activity	Cardiotonic Activity Frog Heart[a] Potency Ratio Ouabain = 1
α,β-Angelica lactone	American Cyanamid, Calco Division		...	4,770
β,γ-Angelica lactone	American Cyanamid, Calco Division		...	600
β-Methyl-α,β-angelica lactone	Synthesized		1:1,000	4,098
β-Methyl-β,γ-angelica lactone	Synthesized		1:1,000	4,098
γ-Methyl-α,β-angelica lactone	Synthesized		1:1,000	...
γ-Ethyl-α,β-angelica lactone	Synthesized		1:1,000	Not tested
γ-Butyrolactone	E. I. DuPont De Nemours Company		...	...
γ-Valerolactone	E. I. DuPont De Nemours Company		...	...
Tertiary-butyl hydroperoxide	Novadel-Agene Corp., Lucidol		1:1,000	1,037
Di-tertiary-butyl peroxide	Shell Development Corp.			
Hydrogen peroxide			...	...
Ouabain			1:100,000	1
Quinine			1:100,000	...

[a] Reference 6.

Although lower temperatures prolonged the life of the muscles, the veratrinic response was abolished below 20° while temperatures higher than 25° reduced the life of the muscles.

Compounds were tested by first stimulating the muscle pairs with the specified stimuli at two-minute intervals. Approximately equal contractions were sought from each pair but were seldom obtained owing to the marked variation even in the paired muscles of a single animal. After the control stimuli one of the muscles was exposed to the desired concentration of the test drug in the bath fluid. Compounds were first tested at a concentration of 1:1,000 and when antiveratrinic activity was demonstrated, at 1:5,000. The addition of the test drug is indicated by the first arrow in the upper record of each pair. Six additional stimuli at two-minute intervals were then administered to both muscles. Pretreatment with the test substance was conducted to determine if the compound produced a direct action on the muscle. Following the twelve minute pretreatment period, both muscles were simultaneously exposed to a 1:10,000,000 concentration of veratridine as indicated by the second arrow in the upper record and the first arrow in the lower control record. Approximately four tests were run with each compound, depending upon the response from the preparations.

RESULTS AND DISCUSSION

While α,β- and β,γ-angelica lactones are potent cardiotonic agents on the frog heart and active peroxide-forming compounds (6), they are inactive as antiveratrinic agents at a concentration of 1:1,000. β-Methyl-α,β-angelica lactone and β-methyl-β,γ-angelica lactone form peroxides and possessed antiveratrinic activity (Fig. 1). Two other unsaturated lactones, γ-methyl-α,β-angelica lactone and γ-ethyl-α,β-angelica lactone, which presumably do not form any appreciable amount of peroxides, nevertheless exhibit an antiveratrinic effect (Fig. 2).

The two saturated γ-lactones, butyrolactone and γ-valerolactone, did not modify the veratrinic response at the maximal concentration of 1:1,000.

Tertiary-butyl hydroperoxide produced a strong initial antiveratrinic action at a concentration of 1:1,000 (Fig. 3), but was inactive at 1:5,000. The corresponding di-tertiary-butyl peroxide in which the peroxide is carbon to carbon was inactive. This

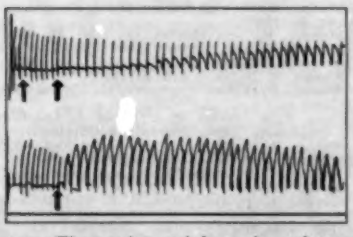


Fig. 1.—The antiveratrinic action of β-methyl-angelica lactone 1:1,000 on paired sartorius muscles of female *Rana pipiens*. First arrow, upper record, β-methyl-angelica lactone. Second arrow upper record and arrow lower record, veratridine 1:10,000,000.

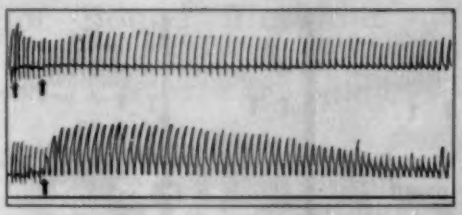


Fig. 2.—The antiveratrinic action of γ-ethyl angelica lactone 1:1,000 on paired sartorius muscles of female *Rana pipiens*. First arrow, upper record, γ-ethyl-angelica lactone. Second arrow upper record and arrow lower record, veratridine 1:10,000,000.

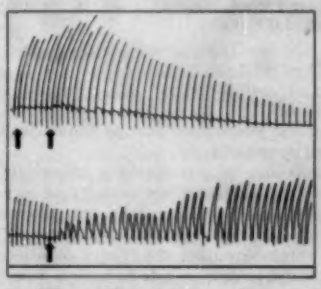


Fig. 3.—The antiveratrinic action of tertiary-butyl hydroperoxide 1:1,000 on paired sartorius muscles of female *Rana pipiens*. First arrow upper record, tertiary-butyl hydroperoxide. Second arrow upper record and arrow lower record, veratridine 1:10,000,000.

corresponds to previously observed activity on the frog heart (6). Hydrogen peroxide at a concentration of 1:10,000 caused contraction of the muscle to such a degree as to prevent observation of possible antiveratrinic action. At a 1:20,000 concentration neither contraction nor antiveratrinic activity was observed.

In order to compare the results with those of Arora (4), his work with ouabain was repeated. Ouabain at concentrations of 1:100,000 induced an almost complete immediate antiveratrinic response analogous to the lactones but lasting much longer. A 1:300,000 concentration required fourteen minutes for the full effect while a 1:1,000,000 concentration required about twenty-five minutes (Fig. 4). A comparison of the inhibition induced by the lactones and peroxides with that resulting from ouabain reveals certain qualitative as well as quantitative differences. The active lactones and peroxides produce a short but immediate reduction or complete abolition of the veratrinic response. This antagonism is transient and the full response is gradually restored. Even after strongly active concentrations of ouabain (1:100,000) the response was never completely blocked at the time the veratridine was first added but in contrast to the lactones the effect lasted indefinitely.

The differences in the responses as described led to the trial of another antiveratrinic agent, quinine. At concentrations of 1:100,000 quinine exhibited the same initial depression in addition to the gradual

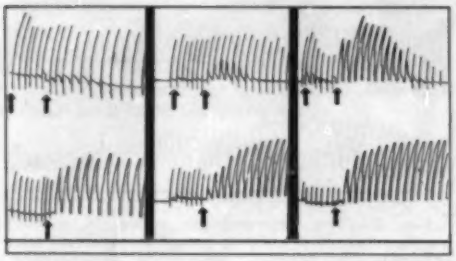


Fig. 4.—The antiveratrinic action of ouabain. First arrow in each upper record, ouabain. Second arrow upper records and arrow lower records, veratridine 1:10,000,000. Left record, ouabain 1:100,000: center record, ouabain 1:300,000; right record, ouabain 1:1,000,000.

return of the veratrinic effect as shown by the lactones and hydroperoxide.

The difficulty of attempting a quantitative estimation of the antiveratrinic activity of any of the compounds was emphasized by the extreme variability of the frog muscles. For example, a few records obtained during the winter months showed tertiary-butyl hydroperoxide to have a trace of antiveratrinic activity at 1:100,000; a slight action at 1:50,000 and a very definite activity at 1:10,000. Later in the year the same compound was consistently found to be completely inactive at 1:5,000. It was observed throughout the study that the antiveratrinic response of a single group of frogs was fairly uniform while the variation between groups of frogs ordered from the same supplier during the same season was highly variable.

It would appear that the lactones are not analogous to the cardiac glycosides in their antiveratrinic action, but instead more closely resemble quinine. More important, the activity of the unsaturated lactones could not be satisfactorily related to their capacity to form peroxides. The two most active peroxide-forming and cardiotonically active lactones, α,β- and β,γ-angelica lactones were inactive although the corresponding β-methyl compounds blocked veratridine. It was noted that α,β- and β,γ-angelica lactones produced a yellow color in the bath fluid when veratridine was added. Lactones with γ,γ-substituents which have no digitalis-like action on the frog heart and which presumably do not form peroxides (6) were active however.

The inactivity of the saturated lactones is not surprising although butyrolactone has been found to be an active antagonist to the cardiotonic action on digitalis. It has been thought that butyrolactone may occupy the same receptors as digitalis (14). If the receptors occupied by digitalis in skeletal muscle are similar to those in the heart, it would appear that butyrolactone might also block the veratrinic response. As indicated this block did not occur. If, however, the antiveratrinic response requires more than occupation of receptor sites, but some additional factor such as peroxide formation, then the saturated lactones would not be expected to reproduce the blocking action of digitoxin. The fact that tertiary-butyl hydroperoxide produces an immediate antiveratrinic response similar to that resulting from the active lactones and that most of these lactones definitely form peroxides (6) makes such formation a possibility. An explanation for the inactivity of α,β- and β,γ-angelica lactones might be deduced from an examination of the structures in Table I. All active experimental compounds are either hydroperoxides or unsaturated lactones with at least 2-carbon substitution. That the degree of alkylation is significant might also be deduced from the observation that γ-ethyl angelica lactone, the most highly alkylated lactone tested, consistently produced a longer depression of the antiveratrinic response than the other lactones. Although the antiveratinically active γ-substituted angelica lactones are not supposed to form peroxides due to lack of resonance (6), it is possible that amounts may be formed (15). On the basis of the limited evidence, however, it must be concluded that although hydroperoxides and some peroxide-forming lactones have antiveratrinic activity this activity cannot definitely be related to their ability to form peroxides or to the antiveratrinic effect of the cardiac glycosides.

SUMMARY

1. Six unsaturated and two saturated γ-lactones, a carbon to carbon peroxide, a hydroperoxide, and hydrogen peroxide were compared with ouabain and quinine for their antiveratrinic action on the frog sartorius muscle.

2. An antiveratrinic response was elicited by some of the unsaturated lactones and the hydroperoxide. This effect occurred more promptly and lasted for shorter period of time than that produced by ouabain. In this respect the active experimental compounds resembled quinine.

3. Antiveratrinic activity could not be related to peroxide formation by the lactones except that only certain unsaturated lactones and a hydroperoxide blocked the veratrinic response. Activity of these unsaturated lactones may possibly be related to the degree of alkylation.

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Studies on the Structure of "Bound" Morphine*

By JAMES M. FUJIMOTO† and E. LEONG WAY

Crystalline "bound" morphine isolated from the urine of addicts was subjected to various physical and chemical tests. On the basis of X-ray diffraction, infrared, ultraviolet, and electrometric data, the compound was identified to be 3-morphine-glucuronide in the zwitterion form.

IN A PREVIOUS COMMUNICATION the isolation and crystallization of conjugated morphine from the urine of human addicts was reported (1). It was suggested that this morphine metabolite is the principal if not the only form of "bound" morphine excreted in the urine. Indirect evidence for the existence of such a metabolite of morphine was presented earlier (2, 3). Subsequently, Woods (4) isolated a morphine conjugate from the urine and bile of dogs and reported it to be morphine-monoglucuronide dihydrate. The present communication describes experiments leading to the establishment of the identity of the "bound" morphine form the urine of human addicts.

EXPERIMENTAL

Two samples of conjugated morphine from different sources, as well as an authentic sample of morphine, were subjected to various physical and chemical tests as described below. One sample of conjugated morphine was obtained from the urine of human addicts[1] in crystalline form as previously described (1) and the other conjugated morphine obtained from dog urine.[2]

The morphine content was measured by the method of Fujimoto, Way, and Hine (5) after effecting the release of free morphine from the conjugated form by acid-pressure hydrolysis. The method, which is highly specific for morphine, depends upon separation of morphine from other phenolic substances by its selective extraction from a strongly alkaline solution, followed by estimating it with a phenolic reagent. The glucuronide content of "bound" morphine was measured by the glucuronic acid method (6) with naphthoresorcinol or that of Dische (7) using carbazole. Either menthyl glucuronide or glucuronolactone were used as the standards. Powder X-ray diffraction patterns (chromium $K\alpha$) were obtained on the dinitrophenol ether derivatives of the morphine liberated from each sample by acid hydrolysis.[3] Preparation and purification of the dinitrophenol ether derivatives were carried out according to the procedure used by Adler and Shaw (8).

Ultraviolet absorption studies on the samples were carried out in acid and in alkali, using a spectrophotometer (Beckman, model DU). The spectra were first obtained on 3 ml. aliquots of each sample dissolved in 0.5 *N* HCl to make a concentration of 154 mcg./ml., following which 0.5 ml. of 50% potassium hydroxide was added and the spectra again determined. Infrared spectra were obtained on samples either in an oil mull or as evaporated residues with a double-beam spectrophotometer (Perkin-Elmer, model 21). The titration curves of the samples were obtained with 14.5 mg. or 2.9×10^{-3} milliequivalents (using 499 as the molecular weight of morphine monoglucuronide dihydrate) of the metabolite dissolved in 5 ml. of distilled water. Higher concentrations of the material were not used because of the low solubility of the compounds. The course of the titration was followed with a pH meter (Beckman, model H-2), adding standard acid and base by means of a microburette. The initial pH was 5.8.

RESULTS

Table I shows that the morphine content of the crystalline bound morphine isolated from urine of human addicts is the same as that

TABLE I.—MORPHINE AND GLUCURONIC ACID CONTENT OF THE MORPHINE METABOLITES ISOLATED FROM HUMAN URINE AND FROM DOG URINE

Test	Metabolite from Human Urine	Metabolite from Dog Urine	Theoretical Values[a]
Morphine Content (% of total weight)	56.8 ± 3.0	56.8 ± 2.2	57.2
Glucuronic Acid Content (% of total weight)	42.8 ± 1.0	41.8 ± 0.4	38.9

[a] From Woods (4) for morphine monoglucuronide dihydrate.

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[1] The urine from addicts was obtained through the courtesy of Drs. Harris Isbell, H. Fraser, and Ann Eisenman, Addiction Research Center, Lexington, Ky.

[2] Kindly furnished to us by Dr. L. A. Woods of the University of Michigan.

[3] We are indebted to Dr. D. H. Templeton of the Department of Chemistry, University of California Berkeley, for the powder X-ray diffraction pattern determinations and interpretations.

for the conjugated morphine obtained from dog urine. The values obtained are consistent with calculated values for the morphine monoglucuronide dihydrate postulated by Woods (4). The presence of morphine as an essential component of conjugated morphine from urine of human addicts was further established by the powder X-ray diffraction studies made on the dinitrophenol derivative of the hydrolyzed morphine conjugate. The pattern (Fig. 1) obtained showed lines corresponding in position and intensity with those obtained from authentic morphine dinitrophenyl ether. The pattern of the latter agrees with those of the ether derivative of authentic and biosynthetic morphine (8). The pattern of the dinitrophenol ether of the hydrolyzed morphine conjugate derived from dog urine was also the same as the other patterns. This procedure is highly specific for morphine and is capable of distinguishing slight differences between ethers of morphine and dihydromorphine where the usual colorimetric methods fail.

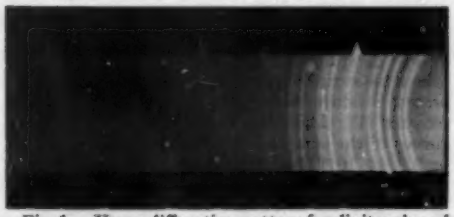

Fig. 1.—X-ray diffraction pattern for dinitrophenyl ether derivation of morphine from crystalline bound morphine.

The presence of the glucuronic acid moiety in the bound morphine rests on two types of evidence. In the infrared curves shown in Fig. 2 the very strong band in the 3μ region is in agreement with the OH stretch frequency broadened by association to be expected from a polyhydroxy glucuronide compound. The strong absorption in the 6 μ–7 μ region is in agreement with the presence of a carboxylate ion. Also, the compound gives the typical color reaction for glucuronic acid by both the naphthoresorcinol and carbazole methods for the estimation of glucuronic acid type compounds. It should be noted, however, that the glucuronide values given in Table I are somewhat higher than theoretical. This is probably attributable to a higher color index for the morphine monoglucuronide as compared with the standards. This is not surprising since higher values for other glucuronic acid conjugates as compared to glucuronolactone are known to exist. In all probability this morphine conjugate is a β-D-glucuronide but this point was not established.

The molecular site of conjugation of bound morphine with glucuronic acid appears to be at the 3-phenolic position since the phenol reagent used in the morphine determination procedure did not give a color with the conjugated material until after acid pressure hydrolysis. Moreover, the ultraviolet absorption characteristics of the compound in acid and base give no evidence as to the presence of a free phenol (Fig. 3). Morphine and its congeners which have a free phenol group at the 3 position (such as nalorphine, normorphine

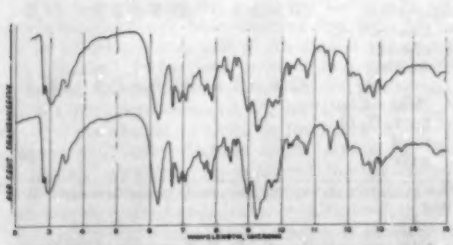

Fig. 2.—Infrared absorption characteristics of bound morphine from human addict urine (upper curve) and from dog bile (lower curve).

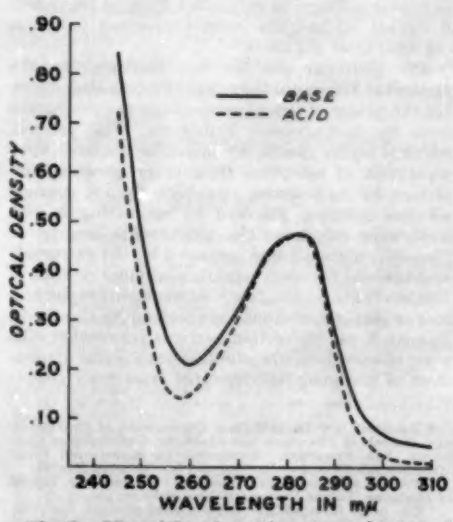

Fig. 3.—Ultraviolet absorption curves for bound morphine in acid and base. Morphine monoglucuronide, 154 microgm./ml. distilled water.

dilaudid, and metopon) show a bathochromic shift with an increase in pH as is generally known for phenols (5). Since the curve for the morphine conjugate is very similar to that obtained for codeine in which the phenol group is masked, the evidence is consistent with the postulate that the conjugation of morphine with glucuronic acid occurs at the 3- position of morphine.

Further evidence to support the conclusion that morphine conjugate from addict urine is a glucuronide was obtained by establishing its identity with morphine monoglucuronide dihydrate obtained by Woods (4) from dog urine and bile. Proof was based on elemental analyses and assays for morphine and glucuronide content. As previously described, our assays of morphine and glucuronide content are compatible with such a structure and, furthermore, the infrared absorption spectra indicate clearly that the two bound morphines from different sources are identical (Fig. 2). The slight difference in the shape of the curves in the region of 3 μ can be explained as due either to the presence of differing amounts of associated water of crystallization or to a minor difference in crystal structure.

The infrared spectra[4] of bound morphine also yield information that indicates the compound exists as a zwitterion. The infrared spectra show a maximum at 6.2 μ with no band between 5.6 and 6.2 μ. This can be interpreted to mean that the carboxyl group of the glucuronic acid moiety is present in an ionized form. Such an interpretation would in turn necessitate the presence of a positive charge on the piperdine nitrogen. Thus the crystal would have to be an internal salt or zwitterion. The possibility that bound morphine might exist as an ampholyte was mentioned by Oberst (9).

The titration curve, Fig. 4, gives additional evidence for the zwitterion formulation as well as an estimate of the pK values for the two groups which make up this zwitterion. Note that there are two pK values, one on each side of the initial starting point of the titration. If these pK values are estimated as occurring at the half-way point of the titration, that is at 1.45×10^{-2} milliequivalents of acid and base, a pK_1 of 3.2 and pK_2 of 8.1 are obtained. The corresponding dissociation constants would then be 6.3×10^{-4} and 0.8×10^{-8}, respectively. Since the latter value is the constant for the conjugate acid, its basic dissociation constant would be 1×10^{-6}. This value is in very good agreement with the value 10^{-6} theoretically predicted by Kumler (10) for the basic dissociation constant of this compound. The value of 6.3×10^{-4} for K_1 is also in excellent agreement with the predicted value of 10^{-4} for the K_d value for this compound (10). K_1 is the dissociation constant for the structure with a positive charge on the nitrogen, while K_d is for the form without a positive charge. According to Kumler (11), K_1 will be somewhat larger than K_d because of the positive charge on the nitrogen. While the positive charge on the nitrogen is acid-strengthening, it could not be responsible for the observed increase of acidity of 0.8 of a pKa unit by its inductive effect through nine intervening atoms. However, molecular models of the compound show that the positively charge nitrogen and the carboxyl group can approach within four Ångströms of one another and the resulting field effect directly through space could account for an increase of acidity corresponding to a few tenths of a pKa unit. Based on Kumler's calculations, at pH 5.8, 99.99% of morphine monoglucuronide dihydrate is in the zwitterion form (10) with the structure as shown in Fig. 4.

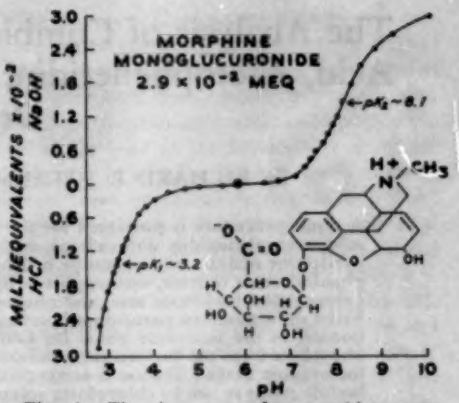

Fig. 4.—Titration curve for morphine monoglucuronide and approximate pK values. Formula for zwitterion.

[4] We are grateful to Dr. L. A. Strait and Mr. Michael Hrenoff, Spectrographic Laboratory, School of Pharmacy, University of California Medical Center, San Francisco, for the infrared determinations and their comments.

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The Analysis of Combinations of Acetylsalicylic Acid, Acetophenetidin, and Caffeine with Other Drugs*

By RICHARD F. HEUERMANN and JOSEPH LEVINE

A rapid procedure is presented for the total analysis of mixtures of acetylsalicylic acid, acetophenetidin, and caffeine, with the following compounds: codeine salts, pyrilamine maleate, thonzylamine hydrochloride, chlorprophenpyridamine maleate, phenindamine tartrate, methapyrilene hydrochloride, doxylamine succinate, cyclopentenylallyl barbituric acid, and phenobarbital. Separation of the components is based on a modified partition chromatographic procedure in which sodium bicarbonate, as the immobile phase on Celite, traps acetylsalicylic acid; tripotassium phosphate traps the barbiturates; sulfuric acid traps caffeine; and tartaric acid traps the organic bases. Elution is accomplished subsequent to neutralization of the immobile phase *in situ* by chloroform solutions of acetic acid or triethylamine, respectively. The concentrations of the separated components are measured spectrophotometrically. An analysis can be completed in approximately one and one-half hours.

ACETYLSALICYLIC ACID, acetophenetidin, and caffeine (APC) are frequently compounded with other drugs to provide the combined therapeutic action of the components. The combination with codeine has long been used, and, since the advent of the antihistaminic drugs, combinations of a number of these with APC have been introduced. Tablets of several barbiturates with APC are also available.

A recent publication (1) describes a rapid chromatographic separation of acetylsalicylic acid, acetophenetidin, and caffeine. With appropriate modification, this procedure can be extended to include the following compounds in combination with APC: codeine sulfate, codeine phosphate, pyrilamine maleate, thonzylamine hydrochloride, chlorprophenpyridamine maleate, phenindamine tartrate, methapyrilene hydrochloride, doxylamine succinate, phenobarbital, and cyclopentenylallyl barbituric acid. Two separate modifications of the original APC assay are required: the first for the tablets containing codeine and the antihistamines, which are organic bases; the second for tablets containing the barbiturates, which are acidic. Several of these compounds are present in such low concentration that samples much larger than that previously designated (1) must be taken to provide a sufficient quantity of the compound for accurate measurement. These larger samples are readily accommodated.

In the procedure for acetylsalicylic acid, acetophenetidin, and caffeine alone, (1) the separation of the components is achieved by passing a chloroform-ether solution of the mixture over a two-segment chromatographic column consisting of Celite as supporting phase, with sulfuric acid as the immobile phase on the first segment and sodium bicarbonate on the second segment. Acetophenetidin passes directly through the column. Caffeine is trapped on the acid segment, from which it is eluted with chloroform. Acetylsalicylic acid is trapped on the sodium bicarbonate segment, from which it is eluted with a chloroform-acetic acid solution, which acidifies the sodium bicarbonate and converts the sodium salt of acetylsalicylic acid to the free acid.

In a manner analogous to the separation of acetylsalicylic acid, it was expected that in mixtures containing the organic bases, the latter would be trapped on the acid segment, but, being more strongly basic than caffeine, would remain trapped during the elution of caffeine by chloroform. After removal of the other components, the sulfuric acid could be neutralized and the bases eluted with a chloroform solution of triethylamine.

This procedure was found to be feasible for a number of the bases; however, leakage occurred in some cases, notably with phenindamine, and to a much lesser extent with pyrilamine. The insolubility of phenindamine tartrate in chloroform suggested that the partial elution of phenindamine in the caffeine and acetylsalicylic acid fractions could be prevented by the use of tartaric acid as immobile phase on Celite. This

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We wish to thank the following for furnishing us with samples to be used as standards: Merck and Company for pyrilamine maleate; The Wm. S. Merrell Co. for doxylamine succinate; Nepera Chemical Company for thonzylamine hydrochloride; Hoffmann-LaRoche, Inc., for phenindamine tartrate; The Upjohn Company for cyclopentenylallyl barbituric acid; Monsanto Chemical Company for methapyrilene hydrochloride, and The Schering Corp. for chlorprophenpyridamine maleate.

was completely effective in trapping both phenindamine and pyrilamine.

It was also found that any acetic acid residue on the column after the elution of acetylsalicylic acid is eluted with the organic base by the triethylamine. Evaporation of the triethylamine acetate requires prolonged heating. Complete removal of triethylamine is necessary since the commercial product, even after careful fractionation, absorbs in the ultraviolet region. This difficulty is circumvented by placing the Celite-acid segments and the Celite-sodium bicarbonate segment in separate columns which are placed in series one above the other. During the application of the sample and the elution of acetophenetidin and caffeine, the effluent from the sodium bicarbonate column is passed directly onto the acid column; the columns are then separated and the acetylsalicylic acid and the organic base are eluted from the individual columns.

The vapor pressure of phenindamine is such that slight losses occur during the evaporation of the excess triethylamine which is present in the eluate. Fortuitously, phenindamine can be eluted with chloroform saturated with ammonia, which is very easily evaporated. This solution fails, however, to elute the other organic bases readily, presumably because of the low solubility of ammonia in chloroform. It is probable that the greater ease in the elution of phenindamine is directly related to the greater difficulty in trapping the material.

Separation of the barbiturates from APC is based upon their weakly acidic nature. Banes (2) separated phenobarbital from acetylsalicylic acid by passage over a column of Celite–disodium phosphate which trapped only the acetylsalicylic acid. In the present procedure, Celite–sodium bicarbonate, which is used to trap acetylsalicylic acid, allows the barbiturates to pass through; these are later quantitatively trapped by the more strongly basic tripotassium phosphate. Here, as in the combinations of APC with the organic bases, two columns in series are used. The upper column containing the Celite-sodium bicarbonate traps the acetylsalicylic acid. The effluent from this column, containing acetophenetidin, caffeine, and the barbiturate, passes directly over the second column containing Celite–tripotassium phosphate and Celite–sulfuric acid, which trap the barbiturate and caffeine and allow the acetophenetidin to pass through. After elution of caffeine, the columns are separated and the acetylsalicylic acid and the barbiturate, respectively, are eluted with a solution of acetic acid in chloroform.

The sample is prepared for introduction onto the column as previously described (1): a weighed portion of the powdered material is transferred to a volumetric flask, mixed thoroughly with chloroform (containing a small amount of acetic acid in the case of the barbiturates or of triethylamine in the case of the organic bases to liberate these compounds from their salts), and adjusted to volume. Without separation of the insoluble excipient material, an aliquot is diluted with ether and passed directly onto the column.

All tablets except those containing phenindamine could be prepared in this manner; low and erratic recoveries were obtained with this product. Complete recovery of phenindamine was achieved, however, by first macerating the powdered material with a small amount of water, adding sufficient absolute alcohol to attain miscibility with chloroform, and making the whole to volume with chloroform containing a small amount of triethylamine.

During the development of the procedure, occasional low recoveries of the organic base were obtained. In these instances, the columns were extruded and strips of indicator test paper laid on the still-formed packing. Small acidic areas were found at the base of the columns; these were absent when quantitative recoveries were obtained. By packing a small segment of Celite-ammonium hydroxide at the base of the column, the incidence of acid areas and accompanying low recoveries was effectively eliminated.

Measurements of the isolated components are made spectrophotometrically. Acetophenetidin, caffeine, and acetylsalicylic acid are prepared for measurement as previously described (1) except for dilutions occasioned by the use of larger samples. The other components are measured in aqueous solution as their salts.

PROCEDURE

(1) **Preparation of Sample.**—Ascertain the average weight per tablet. Grind to a fine powder (in the case of coated tablets grind to pass through a 60-mesh seive) a sufficient number of tablets (*e. g.*, 20 tablets) to provide a representative sample. Transfer to a 50-ml. volumetric flask a weighed sample containing approximately the following amounts: codeine salts, 25 mg.; barbiturates, 25 mg.; thonzylamine, 10 mg.; other antihistamines, 5 mg. Add approximately 25 ml. of chloroform except in the case of phenindamine (see below). To the samples containing barbiturates add 0.3 ml. of acetic acid; to the others add 0.3 ml. of redistilled triethylamine; shake thoroughly, and make to volume with chloroform. In the case of phenindamine, add to the powdered sample in the volumetric flask 1 ml. of distilled water, and completely wet the powder. Allow to stand approximately thirty minutes, then

add 9 ml. of absolute ethyl alcohol and 0.3 ml. of triethylamine, and make to volume with chloroform.

(2) **Chromatographic Column.**—The column is made of a 25 × 200 ml. test tube to the bottom of which is attached an approximately 5 cm. length of 6–8 mm. tubing. The tamping rod consists of a disk of stainless steel, aluminum, or glass, with a diameter 1 mm. less than that of the column, attached to a rod 12 to 18 inches long. Pack fine glass wool in the base of the column to act as a support.

To 2.0 Gm. of Celite 545 (Johns-Manville Corp.) in a mortar add 2.0 ml. of approximately 1 *N* $NaHCO_3$ and incorporate thoroughly by kneading with a flexible spatula blade. Transfer to column and tamp, using gentle pressure, to compress the material to a uniform mass. This constitutes column *A*. In a like manner, mix 1.0 Gm. of Celite with 0.5 ml. of concentrated NH_4OH, and pack in a second column. Above this place a mixture of 2.0 Gm. of Celite with 2.0 ml. of 4 *N* H_2SO_4, followed by 2.0 Gm. of Celite mixed with 2.0 ml. of 1 *N* tartaric acid. This constitutes column *B*, used for APC-organic base combinations. For APC-barbiturate combinations, column C is prepared with a lower segment composed of 2.0 Gm. of Celite with 2.0 ml. of 4 *N* H_2SO_4 and the upper segment containing 2.0 Gm. of Celite with 2.0 ml. of 1 *N* K_3PO_4.

A. Separation of APC-Organic Base Combinations.—(Use water-washed solvents in the ensuing procedure). Mount columns *A* and *B* in such a manner that the effluent from column *A* flows directly into column *B*. Wash with 15–20 ml. of ether, discarding the washings.

Without filtering the sample preparation, withdraw a 5-ml. or 10-ml. aliquot (depending upon the content of the components) and dilute with 4 volumes of ether. Allow solution to pass through columns. After the last portion has passed onto the adsorbent, wash with five 5-ml. portions of ether, allowing each to pass onto the adsorbent prior to the addition of the ensuing one. Evaporate the combined eluate to dryness on a steam bath in a current of air. This fraction contains the acetophenetidin. Dissolve the residue in 5 ml. of chloroform and make to volume with isooctane in a 50 ml. volumetric flask. Make any dilution necessary to adjust to a concentration suitable for spectrophotometric measurement with a 1:10 mixture of chloroform and isooctane.

Immediately after passage of the last portion of ether through the columns, replace the receiver with a 50-ml. volumetric flask. Pass 48 ml. of chloroform through columns and dilute to volume. This fraction contains caffeine, which is determined spectrophotometrically after proper dilution with chloroform.

Separate columns *A* and *B*. Place a 100-ml. volumetric flask as receiver for column A and pass a solution of 0.5 ml. of glacial acetic acid in 5 ml. of chloroform through the column followed by 92 ml. of a 1% solution of acetic acid in chloroform. Adjust to volume and make proper dilutions with a 1% acetic acid-chloroform solution for the determination of acetylsalicylic acid.

Add to column *B* (except in the case of phenindamine) a solution of 2 ml. of redistilled triethylamine in 5 ml. of chloroform followed by 100 ml. of a 1% solution of triethylamine in chloroform. Evaporate the eluate to dryness on a steam bath in a current of air, heating two to five minutes after evaporation of solvent to insure complete removal of the triethylamine. For samples containing phenindamine, elute with chloroform saturated with ammonia, prepared by shaking 100 ml. of chloroform with 25 ml. of concentrated ammonium hydroxide. When evaporating this eluate, remove from heat immediately upon the removal of the last trace of solvent.

Dissolve the residue in 10 ml. of water containing 5 to 10 drops of hydrochloric acid, and adjust to a suitable volume with water for the determination of the organic base.

B. For APC-barbiturate combinations.—Mount columns *A* and *C* as above and proceed as described above for the elution of acetophenetidin, caffeine, and acetylsalicylic acid. For the elution of the barbiturate, add to column C, 0.5 ml. of glacial acetic acid in 5 ml. of chloroform, followed by 95 ml. of a 1% solution of acetic acid in chloroform. Evaporate to dryness. Dissolve the residue in 10 ml. of water containing 10 drops of ammonium hydroxide, and adjust to a suitable volume with water.

In the spectrophotometric determination of all of the ingredients separated, read the absorbances of the solutions prepared as above and compare the values obtained with those simultaneously determined on corresponding standard solutions. Chloroform containing 1% acetic acid is used for solutions of salicylic and acetylsalicylic acids and the latter standard must be prepared daily.

As a guide in determining the proper dilutions, the values in Table I may be used.

RESULTS

Mixtures of acetophenetidin, caffeine, acetylsalicylic acid, and the associated compounds, in ratios approximating those found in the commercial

TABLE I

Compound	Wavelength mμ	Concentration mcg./ml.	Absorbance (Approximate)
Acetophenetidin	285	50	0.505
Caffeine	276	10	0.485
Acetylsalicylic Acid	280	100	0.790
	310	100	0.010
Salicylic Acid[a]	280	25	0.113
	310	25	0.573
Pyrilamine Maleate	315	10	0.205
Chlorprophenpyridamine Maleate	265	10	0.212
Phenindamine Tartrate	260	10	0.219
Methapyrilene Hydrochloride	314	10	0.274
Doxylamine Succinate	261	10	0.215
Thonzylamine Hydrochloride	314	10	0.102
Codeine Sulfate	284	100	0.444
Codeine Phosphate	284	100	0.395
Phenobarbital	240	10	0.445
Cyclopentenylallyl Barbituric Acid	241	10	0.366

[a] Calculated as acetylsalicylic acid. For calculation of total acetylsalicylic acid in mixture with partially hydrolyzed material see (1).

TABLE II

Associated Compound	Acetophenetidin Added Mg.	Acetophenetidin Found Mg.	Caffeine Added Mg.	Caffeine Found Mg.	Acetylsalicylic Acid Added Mg.	Acetylsalicylic Acid Found Mg.
Pyrilamine Maleate	1.00	0.99	10.40	10.49	2.00	1.99	17.00	16.52
	1.00	1.00	11.90	11.89	2.00	2.00	17.30	17.03
Phenindamine Tartrate	1.00	1.00	29.50	29.56	2.00	2.00	38.00	37.59
	1.00	0.98	29.80	30.05	2.00	2.00	29.40	29.11
Thonzylamine Hydrochloride[a]	1.00	1.02	5.00	5.09	2.00	1.99	10.40	10.22
	1.00	1.02	5.00	5.08	2.00	1.97	10.90	10.63
Doxylamine Succinate	1.00	1.01	24.80	24.50	5.00	4.96	40.50	40.51
	1.00	1.02	25.50	25.29	5.00	5.01	49.80	49.87
Methapyrilene Hydrochloride	1.00	1.00	5.00	5.03	2.00	1.98	10.20	10.09
	1.00	1.00	5.00	4.94	2.00	1.98	12.06	11.74
Chlorprophenpyridamine Maleate	0.50	0.51	40.00	40.10	10.00	9.92	62.50	63.70
	0.50	0.50	42.00	42.25	10.00	9.94	60.40	60.00
Codeine Sulfate	2.50	2.55	19.00	18.87	3.00	3.01	31.70	31.08
	2.50	2.49	20.90	21.03	3.00	3.01	24.00	23.29
Phenobarbital	2.50	2.51	25.10	24.86	5.00	4.90	40.30	40.51
	2.50	2.53	25.60	25.05	5.00	4.92	34.60	34.56
Cyclopentenylallyl Barbituric Acid[b]	2.28	2.23	26.00	25.78	5.00	4.80	34.10	34.11
	2.28	2.24	26.30	26.32	5.00	4.94	36.00	36.01

[a] Mixture contains 1 mg. ascorbic acid.
[b] Sodium salt added.

TABLE III

Associated Compound	Declared Mg./Tab.	Found Mg./Tab.	Acetophenetidin Declared Gr./Tab.	Acetophenetidin Found Gr./Tab.	Caffeine Declared Gr./Tab.	Caffeine Found Gr./Tab.	Acetylsalicylic Acid Declared Gr./Tab.	Acetylsalicylic Acid Found Gr./Tab.
Pyrilamine	12.5	12.03	2.50	2.50	0.50	0.50	3.50	3.47
Maleate		12.13		2.45		0.49		3.44
Phenindamine	10.0	9.87	160.0 mg	161.2 mg	15.0 mg	14.84 mg	160.0 mg	160.2 mg
Tartrate		9.86		159.7		14.89		161.5
Thonzylamine	25.0	24.65	1.50	1.46	0.50	0.50	3.50	3.41
Hydrochloride[a]		24.56		1.46		0.50		3.41
Doxylamine	6.0	5.75	2.50	2.31	0.50	0.43	3.50	3.34
Succinate		5.69		2.32		0.44		3.36
Methapyrilene	25.0	24.09	2.50	2.44	0.50	0.47	3.50	3.47
Hydrochloride		24.32		2.43		0.48		3.41
Chloroprophen-	2.0	1.85	2.50	2.53	0.50	0.50	3.50	3.56
pyridamine		1.82		2.52		0.50		3.55
Maleate								
Codeine Sulfate[b]	0.50 gr.	0.49 gr.	2.50	2.48	0.50	0.48	3.50	3.35
		0.48		2.51		0.48		3.38
Codeine	0.50 gr.	0.51 gr.	2.50	2.51	0.50	0.51	3.50	3.49
Phosphate		0.50		2.51		0.50		3.45
Phenobarbital	0.25 gr.	0.25 gr.	2.50	2.49	0.00	0.00	3.50	3.47
		0.25		2.45		0.00		3.50
Cyclopentenyl-	0.75 gr.	0.77 gr.	2.50	2.51	0.50	0.49	3.50	3.43
allyl								
Barbituric Acid		0.78		2.50		0.49		3.47

[a] Contain 20 mg. ascorbic acid per tablet.
[b] Contain flavoring additives.

products, were assayed according to the above procedure. Results are reported in Table II. The assays of commercial tablets are reported in Table III. The duplicate assays represent separate samples, not duplicate aliquots withdrawn from the same sample preparation.

No difficulty was introduced by any of the excipients. In one instance in which the tablet contained a very large proportion of chloroform-insoluble excipient, aliquots were withdrawn both immediately after shaking the sample preparation and after allowing the insoluble matter to precipitate; no difference was found in the results.

SUMMARY

Tablets containing acetylsalicylic acid, acetophenetidin, and caffeine combined with codeine, barbiturates, or antihistaminic drugs may be separated into their component compounds by a modified partition chromatographic procedure. Acetophenetidin, which is neutral, passes through the chromatographic column in chloroform-ether solution. Caffeine, a feebly basic alkaloid, is trapped by sulfuric acid, from which it is eluted

with chloroform. Acetylsalicylic acid is trapped by sodium bicarbonate; the barbiturates, which are weakly acidic, pass through the sodium bicarbonate but are trapped by tripotassium phosphate. Both are eluted with a solution of acetic acid in chloroform. Codeine and the antihistamines are trapped by tartaric acid and sulfuric acid from which they are eluted with a solution of triethylamine in chloroform. The procedure is rapid; an entire analysis can be completed in approximately one and one-half hours.

The concentrations of the components are measured spectrophotometrically.

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Biosynthesis of C^{14}-Reserpine by *Rauwolfia* Plants*

By EDWIN A. PEETS,† ARTHUR R. SCHULERT,† JOHN SKOK,‡ and WILLIAM CHORNEY‡

Rauwolfia **plants were grown in an atmosphere containing $C^{14}O_2$ for the purpose of biosynthesizing randomly labeled C^{14}-reserpine for use in further metabolic studies. Preliminary experiments indicated that the activity appearing in reserpine, expressed as percentage of the total activity fixed by plant, was lowest in plants started from cuttings, higher in old seedling plants and highest in young seedling plants. A total of 64.5 millicuries C^{14} was incorporated into Rauwolfia plants to produce approximately 225 mg. of reserpine containing 98 microcuries of C^{14} with a specific activity of 670 microcuries per Gm. of carbon.**

RESERPINE, the crystalline indole alkaloid extracted from the roots of plants of the genus *Rauwolfia* (1) has tranquilizing effects in psychiatric disorders as well as hypotensive action. For this reason a great deal of interest has been directed to its metabolism and site of action. The use of a C^{14}-labeled form of the drug would greatly facilitate attempts to obtain such information. Investigations have been made using side-chain-labled reserpine (2, 3), but a randomly labeled form would have the advantage of permitting study of the whole molecule, including the ring moiety. Such randomly labeled reserpine was prepared biosynthetically by supplying *Rauwolfia* plants with $C^{14}O_2$.

PROCEDURES

Materials and Facilities.—Four species of *Rauwolfia* (*serpentina*, *hirsuta*, *verticillata*, and *vomitoria*) were obtained from the Plant Introduction Section of the United States Department of Agriculture.[1] The plants were maintained in the greenhouse and grown in soil. They were prepared for use in biosynthesis by washing the soil from the roots, transplanting the plants to sand, and supplying them with nutrient solutions. The biosynthesis of C^{14}-reserpine was carried out by growing the plants in a specially constructed chamber (4) designed for this purpose. The chamber, located in a greenhouse, consists of a steel frame of approximate dimensions of 5 x 7½ x 6 ft., containing glass panes embedded in a CO_2-impervious mastic. A removable panel permits entry and is sealed when in operation. The plants were grown in gravel contained in a stainless steel bed. Nutrient solutions supplied from a stainless steel storage tank by sub-irrigation contained:

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‡ Argonne National Laboratory.
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[1] The authors wish to thank Dr. Bernice Schubert of the United States Department of Agriculture for procuring the *Rauwolfia* plants for them.

$Ca(NO_3)_2$, 0.0045 *M*; KH_2PO_4, 0.0023 *M*; $MgSO_4$, 0.0023 *M*; B, 0.50 p.p.m.; Mn, 0.25 p.p.m.; Zn, 0.05 p.p.m.; Cu, 0.02 p.p.m.; Mo, 0.05 p.p.m.; and Fe, 0.50 p.p.m. added as Sequestrene NaFe. The temperature in the chamber was maintained at 85 ± 1° F. by a constant flow of temperature-regulated water over the outside surfaces. The relative humidity of the chamber atmosphere was maintained at 50 ± 5%. Air samples from the chamber were pumped through monitoring devices; total CO_2 was measured by an infrared gas analyzer and C^{14} activity of the air phase was measured with a vibrating diaphragm-type ionization chamber. The desired specific activity of the air phase CO_2 was maintained by periodic generation of CO_2 from a calculated ratio of $BaC^{14}O_3/BaC^{12}O_3$. The CO_2 concentration within the chamber was held near that normally present in the atmosphere, namely within the range of 0.01 to 0.06%.

Harvest, Assay, and Isolation Procedures.—At the termination of a designated culture period in a $C^{14}O_2$ atmosphere, the plants were removed from the chamber and the roots were carefully recovered from the gravel substrate. Plant parts were separated, dried at 70° C. in a forced air oven, and ground in a Wiley mill to pass a 30-mesh screen. Samples were counted at infinite thickness with an end-window counter and their radioactivity was determined by comparing the count rate with that of a standard plant sample. The standard consisted of C^{14}-labeled ground plant material, an aliquot of which was assayed by combustion and gas counting.

One to three-Gm. ground root samples were refluxed with 20 ml. of methanol for one hour, filtered and washed twice with hot methanol. The filtrate was partially evaporated *in vacuo* to reduce the volume and made up to 5 ml. Chromatographic examination of this extract using the methods of Banes, *et al.* (5, 6), and Carol, *et al.* (7), established the presence of five fluorescent alkaloids in addition to reserpine, namely deserpidine, rescinnamine, ajmalicine, ajmaline, and serpentine. Reserpine was separated by means of liquid-liquid partition chromatography on Celite columns (6) and assayed by ultraviolet absorption (8). Radioactivity of reserpine was determined by plating the material on a planchet, counting an infinitely thin layer in a windowless gas flow counter, and comparing its count rate with that of a similarly prepared sample of known activity.

The isolation of the labeled material was accomplished by means of a solvent extraction procedure of Korzun, *et al.* (9), rather than by chromatographic methods.

RESULTS

Preliminary Trials.—Preliminary experiments were carried out for the purpose of determining whether there were any species or plant-type differences in capacity for carbon fixation or in efficiency of incorporation of C^{14} into reserpine. Moderately mature and woody seedlings of *R. serpentina* and *R. hirsuta*, young seedlings of *R. verticillata* and young rooted cuttings of *R. verticillata* were selected. They were grown for thirteen days in an atmosphere maintained at an average specific activity of 524 microcuries per Gm. carbon in the air phase. At the end of this period the plants were harvested, and stem, leaf and root fractions were prepared for assay in the manner described. Determinations of reserpine content and activity were also made.

The reserpine content of the roots was similar in all plants and ranged from 0.15 to 0.19%. However, the C^{14} appearing in reserpine, expressed as percentage of the total C^{14} fixed by the plant, varied: smallest amounts appeared in reserpine from roots of plants started from cuttings, higher amounts appeared in old seedlings and considerably higher amounts in young seedling plants. This may be related to the relative rate of root tissue formation and leaf respiration rates by the different types of plants. Young seedling plants are perhaps more vigorous and produce new root tissue at a higher rate than do either older seedlings or cuttings. On the basis of this information young seedling plants were used in the biosynthesis operation.

Biosynthesis of C^{14} Reserpine.—Twelve young seedlings of *R. hirsuta* and two of *R. vomitoria* were transplanted into the chamber and were permitted to become established for a one week period prior to incorporation of $C^{14}O_2$. They were grown for a total of forty days in a $C^{14}O_2$ atmosphere with a specific activity of 1.45 millicuries per Gm. carbon. A total of 64.5 millicuries of C^{14} was fixed by the plants during this time. After a culture period of about thirty-three days under these conditions the *R. vomitoria* plants showed some radiation damage as evidenced by leaf mottling.

Of the 64.5 millicuries of C^{14} fixed by the plants, 46.0 millicuries were taken up by the 12 *R. hirsuta* plants and 18.5 millicuries by the two *R. vomitoria* plants. Of the total amount in the *R. hirsuta* plants, 49.0% appeared in the leaves, 27.7% in the stems and 26.3% in the roots. In the *R. vomitoria* plants, amounts in the comparable fractions were 33.1, 19.4 and 47.1% respectively.

A total of 75 Gm. of dry root material was obtained from the entire crop. Assays of samples of the pooled root material indicated an average reserpine content of 0.3%. The specific activity of reserpine from this pool is 670 microcuries per Gm. of carbon, giving a total available amount of 225 mg. reserpine with a total activity of 98 microcuries of C^{14}.

The reserpine was not all isolated at the same time. Isolations will be carried out as the material is needed for metabolism studies, since the material is apparently less subject to decomposition while in its natural form in the root. The labeled alkaloids are presently being used in animal studies by the first two authors.

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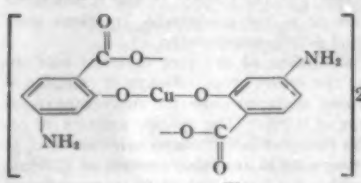
Metal Chelates and Antitubercular Activity III.*

p-Aminosalicylic Acid: Chelate *vs.* Complex

By WILLIAM O. FOYE and RONALD N. DUVALL†

Cupric and ferrous chelates of *p*-aminosalicylic acid have been isolated and characterized, and their physical and antitubercular properties compared with those of a previously isolated cupric complex. The cupric chelate showed much higher *in vivo* antitubercular activity in mice than the cupric complex, and was found to be about thirty times more fat-soluble than the cupric complex. The ferrous chelate was not appreciably active against experimental tuberculosis. The effect of these findings in explaining a possible mechanism of the antitubercular activity of *p*-aminosalicylic acid is discussed.

A COPPER COMPLEX of *p*-aminosalicylic acid (PAS) was isolated by Erlenmeyer, *et al.* (1), and was found to show equivalent or better tuberculostatic activity than PAS itself in two different culture media. The activity of both the complex and PAS was increased tenfold in the presence of excess cupric ion, however, which provides very good indication that PAS exerts its tuberculostatic action in the form of the cupric complex. The complex was believed to be cyclic, or a chelate, although later evidence (2) showed it to be an open phenolic or amine complex (I) rather than a cyclic chelate. At the same time, antitubercular tests in mice showed the complex to be less effective than PAS.



(I)

During the preparation of the copper complex, a green-black product is first visible and then changes gradually to the light green complex in alkaline media. The dark green product was isolated and postulated by Erlenmeyer as a salt, but the evidence presented here indicates it to be a copper chelate. A similar preparation using ferrous ion also produced a compound differing from the previously-prepared ferrous complex (2) and which chemical and biological evidence also indicates to be a chelate structure.

Antitubercular tests in mice, carried out at the Lilly Research Laboratories, showed a striking difference between the PAS complexes and PAS chelates. The copper chelate, when fed to tubercular mice as previously described for the complexes (2), was considerably more active than the copper complex and is superior in activity to PAS itself. The ferrous chelate, although not appreciably active, was much less toxic than the ferrous complex and is, in fact, relatively nontoxic. It was found to have an acute oral toxicity of over 2 Gm./Kg. in mice. The copper chelate, however, killed mice at a dose of 41.7 mg./Kg. but not at 20.8 mg./Kg. after intraperitoneal injection. Detailed results of this testing are presented in Table I.

TABLE I.—IN VIVO ANTITUBERCULAR ACTIVITIES OF THE PAS-METAL CHELATES[a]

Compound	% in Diet	No. of Mice	Mortality, %	Mean Survival Time in Days[b]
$(PAS)_2Cu$	0.5	5	0	23+
$(PAS)_2Cu$	0.01	5	20	22.8
INH[c]	0.01	10	0	23+
Control	..	10	60	21+
$(PAS)_2Fe$	0.1	5	60	20.6+
$(PAS)_2Fe$	0.02	5	80	20.6+
PAS[c]	0.5	5	0	21+
Control	..	10	80	20.3+
PAS[c]	0.5	10	30	20.2+
INH[c]	0.01	10	0	21+
Control	..	10	80	19.5+

[a] Determined at the Lilly Research Laboratories by W. B. Sutton.

[b] A + indicates that the surviving animals were sacrificed at the indicated times for examination of lesions.

[c] PAS = *p*-aminosalicylic acid; INH = isonicotinyl hydrazide.

The method of preparation of the PAS chelates followed that for the PAS complexes already described (2), except that the reactions were allowed to become acidic, thus allowing the isolation of the initially-formed dark colored precipitates. Evidence for chelation, according

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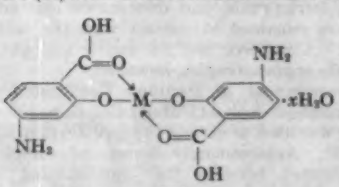
to the criteria listed by Martell and Calvin (3), included: a marked drop in pH during the reaction, absence of metal ions in solution, decreased aqueous solubility, intensification of color, and analytical results in good agreement with a chelate structure having a 2:1 ratio of PAS to metal. Saturated aqueous solutions of the chelates gave negative tests for the metal ions using either ferrocyanide, ferricyanide, or thiocyanate indicators. A positive test was given by the copper complex (I) with ferrocyanide ion, however. Suspensions of the chelates, after standing in dilute hydrochloric acid at a pH of 3.0 also gave negative tests for metal ions.

Elemental analyses were carried out on the air-dried products and the results are presented in Table II along with the corresponding information for the copper complex. It should be pointed out that the variable water contents of these compounds, caused by variations in drying times and procedures, make it difficult to secure exact analytical values. The metal content was determined by ashing at 1,000°, and the water analysis was accomplished both by the Karl Fischer procedure and by loss of weight after vacuum drying at 100°. Unlike the PAS complexes, no sodium ion was present in the chelates. Although an alkaline reaction medium was employed in their preparation, the chelates were isolated at pH values of 4.0 and 5.7, respectively, for the copper and iron derivatives. These results permit the postulation of the following structure (II) for a divalent metal chelate of PAS.



(II)

The ultraviolet absorption spectral characteristics of PAS and its copper derivatives were compared in an attempt to show further differences between the chelate and complex. The position of the maxima observed for PAS itself remained unchanged on formation of either the complex or chelate, but the intensity of absorption (per salicylate ring) increased slightly at 265 mμ and decreased slightly at 300 mμ (see Table III). Little difference in spectral characteristics was noted, however, between chelate and complex. The failure of chelate or complex formation to appreciably alter the position of the maxima has also been noted with the metal chelates of riboflavin (4).

Examination of another physical property, the oil-water partition coefficient, was made not only to discover differences between the chelate and complex, but also to obtain information regarding the penetrability of the metal derivatives into the cells. Oleyl alcohol was selected for this purpose in preference to the more viscous vegetable oils, in accordance with its properties as described by Albert (5). It has also been shown by Collander (6) that the distribution of a series of substances between water and poorly miscible liquids always follows the same order,

TABLE II.—ANALYSES OF THE PAS-METAL CHELATES[a]

Compound	Formula[b]		Analyses, % C	H	N	M	H_2O, % K. F.[c]	D[d]
$(PAS)_2Cu$ complex	$C_{14}H_{10}N_2O_6CuNa_2 \cdot 4H_2O$	Calcd.	34.75	3.72	5.80	13.13	14.89	
		Found	34.94	3.55	5.80	14.89	15.45	15.15
$(PAS)_2Cu$ chelate	$C_{14}H_{12}N_2O_6Cu \cdot H_2O$	Calcd.	43.58	3.63	7.26	16.47	4.67	
		Found	43.48	3.80	7.37	15.81	4.85	5.25
$(PAS)_2Fe$ chelate	$C_{14}H_{12}N_2O_6Fe \cdot 4H_2O$	Calcd.	38.92	4.63	..	12.93	16.67	
		Found	39.29	3.43	..	12.31		15.44

[a] The carbon-hydrogen analyses were determined at the Weiler and Strauss Microanalytical Laboratory, Oxford, England. The nitrogen analyses were obtained by the Kjeldahl method.
[b] The compounds were dried at room temperature by exposure to air to avoid loss of water of hydration as far as possible.
[c] K. F. refers to the Karl Fischer determination.
[d] D refers to vacuum drying at 100° over phosphorus pentoxide.

TABLE III.—ABSORPTION SPECTRAL CHARACTERISTICS

Compound	λ Max., mμ[a]	ε Max.
PAS	265	12,500
	300	9,075
$(PAS)_2Cu$ complex	265	27,290
	300	17,470
$(PAS)_2Cu$ chelate	265	26,490
	300	17,220
$(PAS)_2Fe$ chelate	265	26,880
	300	18,320

[a] The spectra were determined in water using a Beckman Model DU quartz spectrophotometer. Concentrations of approximately 10 mg. per liter were used.

although greater differences in partition coefficients within a given series are obtainable with the liquids of lower water solubility. The coefficients were obtained by measuring spectrophotometrically the percentage of compound extracted by oleyl alcohol from 10^{-5} to 10^{-4} *M* aqueous solutions. At this low concentration, the values observed can, of course, be no more than approximate. Reference to Table IV shows that the copper chelate is considerably more fat soluble (about thirty-fold) than the copper complex, as might be expected from their relative antitubercular activities. The fact that liposolubility is necessary, but is not alone a determinative factor in antitubercular activity, is shown by the comparable liposolubility of the ferrous to the cupric chelate and the relative lack of antitubercular properties by the ferrous chelate.

TABLE IV.—OIL-WATER PARTITION COEFFICIENTS AT 25°

Compound	Concn., Aq. Phase × 10^3 *M*	pH Aq. Phase	Partition Coeff. × 10^3: Oleyl Alc./H_2O
$(PAS)_2Cu$ complex	5	9.8	8
$(PAS)_2Cu$ chelate	6	5.2	244
$(PAS)_3Fe$ chelate	10	4.7	174

It may be stated, on the basis of the evidence presented here and elsewhere (1), that PAS probably exerts its antitubercular action as a cupric chelate. Both *in vitro* and *in vivo* tests have shown the copper derivative to be equal to or superior to PAS itself. That the cupric chelate and not the cupric complex is successful against experimental tuberculosis is undoubtedly due to the much greater lipophilic character of the chelate which allows greater penetration of the cells. The increased *in vitro* activity of both PAS and the copper complex in the presence of excess copper ion points to the possibility that a 1:1 chelate may be the important species; and the fact that doses of the copper chelate comparable to those of PAS were effective in mice presents a strong likelihood that a chelate is the active antitubercular form of this agent. While the chelates were too insoluble to permit the determination of stability constants by customary methods, their stability in water at a pH of 3.0 over prolonged periods would indicate an ability to remain intact in the body. The failure of the ferrous chelate to show appreciable *in vitro* antitubercular activity, whereas both the cupric and ferrous chelates of 8-hydroxyquinoline are known to be the active bacteriostatic forms of this drug (7), and its comparable liposolubility to the cupric chelate, indicate a high degree of selectively toxic action in the antitubercular activity of *p*-aminosalicylic acid chelates.

EXPERIMENTAL

Preparation of the Metal Chelates.—An aqueous solution of copper sulfate (0.05 mole) was added slowly with stirring to a solution of sodium *p*-aminosalicylate (0.1 mole) in 100 ml. of water. The pH dropped from 9.5 to 4.0, as determined by a Beckman pH meter, and a green-black precipitate formed immediately. The suspension was stirred for an hour, filtered, and the product was washed free of sulfate ion with cold water. The dark green product was allowed to dry by exposure to air at room temperature, and a yield of 95% was obtained, based on the formula proposed for the copper chelate in Table II.

The ferrous chelate was prepared in identical fashion, using 0.025 mole of ferrous sulfate and 0.05 mole of sodium *p*-aminosalicylate. The pH dropped from 9.5 to 5.7 during this reaction. A dark red-brown precipitate was isolated in 48% yield, based on the formula in Table II. This compound was appreciably more water-soluble than the copper chelate, and a sizable portion was lost during the washing.

Analysis of the Metal Chelates.—The metal content of the chelates was determined by ashing to constant weight at 1,000°. The ash from the copper chelate was weighed as cupric oxide and that from the ferrous chelate as ferric oxide, and the results were reproducible. The copper complex could not be analyzed by this method because of the presence of sodium carbonate in the ash, so the copper was determined by the standard iodometric procedure. The usual method for analyzing ferrous ion by dichromate oxidation could not be used for the ferrous chelate, since the PAS interfered and no endpoint was visible.

Analysis of the water content was accomplished by the Karl Fischer method using platinum electrodes and a Beckman pH meter. Vacuum drying (10–20 mm.) to constant weight at 100° over phosphorus pentoxide gave comparable values for water to those obtained from the Karl Fischer procedure, but less drastic drying conditions gave variable results.

The chelates were found to be stable at a pH of 3.0 by suspending the chelates in dilute hydrochloric acid and testing for metal ion with nonchelating indicators. Negative tests for metal ion were obtained with each of the chelates using ferrocyanide, ferricyanide, and thiocyanate ions after the chelates remained in contact with the acid overnight. A positive test for cupric ion was found with the copper complex, however.

Measurement of Partition Coefficients.—Commercial oleyl alcohol (DuPont Co., b. p. 137–142°/2 mm.) was used to extract the complexes from water at 25°. Approximately 5 mg. of complex was equilibrated between the aqueous and organic phases by stirring the suspensions for three hours,

by which time all the solid had dissolved. A liter of water was required to dissolve these amounts, giving 10^{-3}–10^{-4} *M* aqueous solutions of chelate or complex, and an equal volume of oleyl alcohol was used. After separation of the layers, residual droplets of oil were removed by filtration through cotton. The concentration of the compound in the aqueous phase was determined spectrophotometrically, and the percentage of compound in each phase was calculated.

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Metal Chelates and Antitubercular Activity IV.*

Isonicotinyl Hydrazide

By WILLIAM O. FOYE and RONALD N. DUVALL†

Metal chelates of isonicotinyl hydrazide (INH) containing divalent copper, cobalt, iron, and zinc and having both 1:1 and 2:1 ratios of INH to metal have been isolated and characterized. These chelates showed *in vivo* antitubercular activities in mice comparable to that from INH itself. The 2:1 chelates were found to be considerably more lipophilic (fifty to ninety-fold) than the 1:1 chelates, but underwent conversion to 1:1 chelates at equilibrium. Toxicity determinations on the chelates showed them to resemble the corresponding inorganic salts in their toxic manifestations in mice. These findings suggest that the INH chelates are present mainly as the 1:1 species *in vivo*, and permit the postulation that INH exerts its antitubercular action as a 1:1 metal chelate.

A TENFOLD INCREASE in the tuberculostatic action of isonicotinyl hydrazide (INH) in the presence of cupric ion was reported by Erlenmeyer, *et al.* (1), on the basis of *in vitro* tests. It was also shown that INH was capable of chelating copper ion giving a 1:1 ratio of INH to copper, and it was suggested that chelation of heavy metal ions played an important part in the antitubercular action of this drug. A chelate structure having a 2:1 ratio of INH to copper was also postulated by Fallab and Erlenmeyer (2), and Albert (3), as well, showed that both 2:1 and 1:1 chelates of INH were capable of existence. Albert also revealed that other hydrazides have equal or greater affinities for metal ions than has INH, but they generally show much lower antitubercular effects (4).

Cymerman-Craig and Rubbo (5) have also presented evidence that INH exerts its tuberculostatic action by means of a metal ion chelation. This was accomplished by blocking the ability of INH to chelate and showing loss of antitubercular activity *in vitro*. These investigators later tested the 1:1 cupric chelate of INH *in vivo* using an ulcer healing technique in mice, and described the chelate to be as active as INH or any of its derivatives, although it showed both local and systemic toxicities (6). They believed the copper chelate to be the active form of the drug *in vivo*.

In this communication, the preparation of several divalent metal chelates of INH and their *in vivo* antitubercular activities are reported. Chelates having a 1:1 ratio of INH to metal (I) were isolated from acidic media, and chelates having a 2:1 ratio (II) were isolated on the alkaline side of neutrality. Coprecipitation of metal hydroxide in the latter reaction was avoided by keeping the pH from exceeding 7.5. The usual indications of chelation were noted, including a marked drop in pH, considerable decrease in aqueous solubility, formation of colored products, and elemental analyses which agree with theoretical values for chelate structures. Chelates (1:1), with cupric, ferrous, and zinc ions, and 2:1 chelates with cupric and cobaltous ions were isolated, and the analytical results are recorded in Table I. An insoluble 1:1 cobalt chelate could not be isolated.

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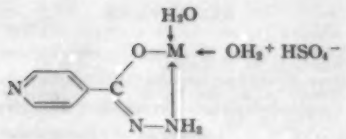
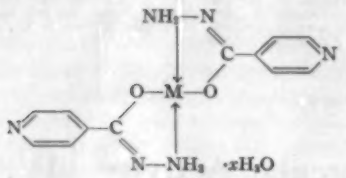
The authors are deeply indebted to Drs. K. K. Chen, R. C. Rathbun, and W. B. Sutton of the Lilly Research Laboratories for their cooperation in carrying out the antitubercular and toxicity tests and providing the results for publication.



(I)



(II)

When these chelates were fed to mice infected with *M. tuberculosis* H37Rv, as previously described for complexes of *p*-aminosalicylic acid (7), their activities were comparable to that of INH itself. Comparison of the relative survival times and mortality percentages of mice protected by the chelates with those of INH-fed mice and unprotected controls would indicate the chelates to be as effective as INH. This becomes evident on comparing the amounts of INH present in the chelates with the amount of uncombined INH used as comparison standard; since 0.025% of the 1:1 copper chelate, for instance, should give the same effect as the 0.01% INH used as standard, based on molecular weights. Histological examination of the mice, however, revealed that a 0.5% dosage level of the 1:1 copper chelate was less effective than a 0.01% level of INH. Detailed results of this testing, carried out at the Lilly Research Laboratories, are shown in Table II.

Determination of toxicities of the INH chelates in mice was also done at the Lilly Research Laboratories, and the results are recorded in Table III. Comparison of these toxicities with those from corresponding amounts of the inorganic salts shows close similarities. Other biological effects of the chelates, such as decrease of activity of the mice, decrease of respiration, skin color, and abdominal tone loss, were also closely paralleled by the injection of the corresponding metal ions as salts. These results indicate that the chelates are decomposed to some extent *in vivo*, and that metal ions are liberated and produce toxic effects. However, the 1:1 chelates are more toxic than would be expected from their metal contents. Whether this decomposition occurs before or after the antitubercular action of the chelate is, of course, not evident from this method of examination. Albert (3) has measured the stability constants of INH-metal

TABLE II.—IN VIVO ANTITUBERCULAR ACTIVITIES OF THE INH-METAL CHELATES[a]

Chelate	Diet, %	No. of Mice	Mortality, %	Mean Survival Time, days[b]
INH-$Cu^+HSO_4^-$	0.5	5	0	21+
	0.2	5	0	21+
	0.1	5	0	21+
	0.02	5	0	21+
	0.01	5	20	20.9
$(INH)_2Cu$	0.1	5	0	21+
	0.02	5	0	21+
$(INH)_2Co$	0.1	5	0	21+
	0.02	5	0	21+
INH-$Zn^+HSO_4^-$	0.2	5	0	21+
	0.1	5	0	21+
	0.02	5	0	21+
INH-$Fe^+HSO_4^-$	0.1	5	0	21+
	0.02	5	0	21+
IHN	0.01	5	0	21+
Controls		10	80	20.3+

[a] Determined at the Lilly Research Laboratories by W. B. Sutton.
[b] A+ indicates that the surviving animals were sacrificed at the indicated times for examination of lesions.

TABLE I.—ANALYSES OF THE INH-METAL CHELATES[a]

Chelate	Formula[b]		% C	% H	% S	% M	H_2O, % K. F.[c]	H_2O, % D[d]
INH-$Cu^+HSO_4^-$	$C_6H_7N_3O_5SCu \cdot 2H_2O$	Calcd.	21.65	3.31	9.62	19.10	10.82	
		Found	22.00	3.25	9.07	18.25	11.47	11.97
$(INH)_2Cu$	$C_{12}H_{12}N_6O_2Cu \cdot 3H_2O$	Calcd.	36.97	4.62		16.30	13.86	
		Found	37.21	3.92		18.03		14.57
$(INH)_2Co$	$C_{12}H_{12}N_6O_2Co \cdot 4H_2O$	Calcd.	35.71	4.96		14.62	17.88	
		Found	35.81	3.89		14.44	13.90	14.09
INH-$Zn^+HSO_4^-$	$C_6H_7N_3O_5SZn \cdot 2H_2O$	Calcd.	21.52	3.29	9.57	19.54	10.76	
		Found	21.58	2.96	9.22	18.24	13.42	12.44
INH-$Fe^+HSO_4^-$	$C_6H_7N_3O_5SFe \cdot 2H_2O$	Calcd.	22.15	3.38	9.85	17.18	11.08	
		Found	21.85	3.15	9.27	15.93	12.08	10.14

[a] The carbon-hydrogen analyses were determined at the Weiler and Strauss Microanalytical Laboratory, Oxford, England.
[b] The compounds were dried at room temperature by exposure to air to avoid loss of water of hydration as far as possible.
[c] K. F. refers to the Karl Fischer determination.
[d] D refers to vacuum drying at 100° over phosphorus pentoxide.

TABLE III.—TOXICITIES OF THE INH-METAL CHELATES[a]

Compound	Dose, I. P., mg./Kg., in Mice[b] 10	25	50	100	250	500	1,000
$INH\text{-}Cu^+ HSO_4^- \cdot 2H_2O$	..	3–4 days	5 hr.	1 hr.	...	...	..
$(INH)_2Cu \cdot 3H_2O$	..	..	1–2 days	3 hr.	23 min.- 1 day	...	..
$CuSO_4 \cdot 5H_2O$	2 days	40 min.- 1 day	1–1½ hr.	40 min.	...	...	..
$INH\text{-}Fe^+ HSO_4^- \cdot 2H_2O$	..	..	..	..	1 day	3 hr.	2 hr.
$FeSO_4 \cdot 7H_2O$	..	..	..	..	3 days	2 days	4 hr.
$(INH)_2Co \cdot 4H_2O$	..	..	..	..	...	1 day	..
$Co(NO_3)_2 \cdot 6H_2O$	..	..	..	..	1 day	9 min.- 1 day	4–6 min.
$INH\text{-}Zn^+ HSO_4^- \cdot 2H_2O$	..	..	..	1–2 days	...	...	..
$ZnSO_4 \cdot 7H_2O$	..	..	..	5 days	17 min.- 2 hr.	17–19 min.	..

[a] Determined at the Lilly Research Laboratories by R. C. Rathbun.
[b] Generally three mice were used for each determination. The times recorded indicate that required for death.

chelates and shown their stabilities to be only slightly less than those of the common amino acid chelates, which chelates are considered to be capable of existence *in vivo* (8, 9).

The ultraviolet absorption spectral characteristics of INH and the divalent metal chelates prepared are listed in Table IV. Little change in the position of maximum absorption for INH took place on formation of the chelates, as was previously observed for the chelates of *p*-aminosalicylic acid (10); but the intensity of absorption decreased with each chelate except the ferrous, when compared with the value to be expected from the amount of INH present. The 2:1 chelates could be distinguished from the 1:1 chelates by the nature of the absorption curves, however. The 2:1 chelates showed pronounced minima at 243–245 mμ, whereas the 1:1 chelates gave flatter curves with no pronounced minima. Prolonged stirring (three to four hours) was necessary to dissolve appreciable amounts of most of these chelates in water, but no attempt was made to reach equilibrium conditions. Essentially the same characteristics were found for the zinc chelate which dissolved at once.

TABLE IV.—ABSORPTION SPECTRAL CHARACTERISTICS

Compound	λ Max., mμ[a]	ε Max.
INH	264	4,780
$(INH)_2Co$	265	7,660
$(INH)_2Cu$	265	6,380
$INH\text{-}Cu^+ HSO_4^-$	265	3,920
$INH\text{-}Zn^+ HSO_4^-$	262.5	4,520
$INH\text{-}Fe^+ HSO_4^-$	262	6,070

[a] The spectra were determined in water at concentrations of 10 mg. per liter.

Examination of the oil-water partition coefficients (Table V) of the INH chelates shows the 2:1 chelates to be fifty to ninety-fold more fat soluble than the 1:1 chelates at the concentrations measured. Oleyl alcohol was used for this determination, in accordance with the advantages outlined by Albert (11). The coefficients were obtained by measuring spectrophotometrically the percentage of compound extracted by oleyl alcohol from 10^{-4} *M* aqueous solutions when freshly prepared. An attempt to reach equilibrium conditions is shown in Table VI. A gradual conversion to an equilibrium, expected to exist between INH and the 1:1 and 2:1 chelates in accordance with Albert's stability constants (3) was apparent after two weeks, with most of the chelate present as the 1:1 species.

TABLE V.—OIL-WATER PARTITION COEFFICIENTS, 25°

Chelate	Concn., Aq. Phase × 10^5 *M*	pH Aq. Phase	Partition Coeff., Oleyl Alc./H_2O
$INH\text{-}Cu^+ HSO_4^-$	15	4.9	0.109
$INH\text{-}Fe^+ HSO_4^-$	15	5.1	0.155
$INH\text{-}Zn^+ HSO_4^-$	15	6.3	0.144
$(INH)_2Cu$	0.75	6.3	10.11
$(INH)_2Co$	1.1	5.8	5.54

TABLE VI.—OIL-WATER PARTITION COEFFICIENTS AFTER TWO WEEKS, 25°

Chelate	Concn., Aq. Phase × 10^5 *M*	pH Aq. Phase	Time	Partition Coeff., Oleyl Alc./H_2O
$INH\text{-}Cu^+ HSO_4^-$	15	4.9	3 hr.	0.109
	15	4.9	2 wks.	0.088
	3.0	6.1	3 days	0.134
	3.0	6.1	2 wks.	0.089
$(INH)_2Cu$	0.75	6.3	3 hr.	10.11
	0.75	6.3	1 wk.	0.222
	0.75	6.3	12 days	0.285
	0.75	6.3	2 wks.	0.167

The antitubercular testing results also indicated a similar shift to 1:1 chelate in the animal body, since the same relative activity was shown by the 2:1 as by the 1:1 chelates.

It is apparent from the much greater lipophilic character of the 2:1 chelates that they should penetrate the cells more readily than the 1:1 chelates (12). Albert (11) has previously demonstrated, for instance, that lowering the liposolubility of 8-hydroxyquinoline analogs and presumably that of their metal chelates as well, considerably reduced or removed their antibacterial activity. In the present case, no difference in antitubercular activity was noted between the 1:1 and 2:1 chelates and INH itself, which in addition to the toxic effects, suggests that the 2:1 chelates are undergoing conversion mainly to 1:1 chelates. In fact, it is doubtful that any appreciable amount of 2:1 chelate would be produced in the body after administration of free INH due to the necessity for alkaline conditions for its formation, and the direction which the equilibrium takes in neutral or acid media. In view of the previous findings regarding the tuberculostatic action of INH chelates (1, 4, 6), it appears from the evidence presented here that INH tends to act as a 1:1 metal chelate, since otherwise chelate formation would be expected to reduce the antitubercular activity of INH, and both the 2:1 chelate and free INH should be readily converted to the 1:1 chelate under biological conditions.

EXPERIMENTAL

Preparation of the Metal Chelates.—The 1:1 chelates were prepared by adding an aqueous solution of the metal sulfate (0.02 mole) slowly with stirring to 2.7 Gm. (0.02 mole) of isonicotinyl hydrazide[1] in 50 ml. of water. Precipitation took place with in a few minutes, and the pH dropped from 7.1 to 3.2 during the addition of copper sulfate, from 7.1 to 5.9 using zinc sulfate, and from 7.2 to 4.8 with ferrous sulfate. The mixtures were stirred for one hour, filtered, and washed with water. The products were dried by exposure to air at room temperature. A 92% yield of pale green copper chelate, an 83% yield of white zinc chelate, and an 87% yield of bright orange ferrous chelate were isolated, based on the formulas proposed in Table I.

The 2:1 chelates were obtained by the dropwise addition of aqueous solutions of copper sulfate and cobaltous chloride (0.01 mole) to 0.02 mole of isonicotinyl hydrazide[1] in 50 ml. of water while maintaining the pH at approximately 7.5. After being stirred for an hour, the mixtures were filtered, washed with water, and air dried. An 83% yield of green copper chelate and a 72% yield of pink cobalt chelate were isolated. With both the 1:1 and 2:1 chelates, no further purification was done prior to analysis in order to avoid loss of water of hydration and avoid, as a result, products of varying composition.

Analysis of the Metal Chelates.—Metal analyses were done by ashing at 1,000° as reported earlier (10). Copper was also determined by the standard iodometric procedure. Sulfur was determined by dissolving the 1:1 chelates in hydrochloric acid T.S. and precipitating the sulfate as barium sulfate. Water of hydration was measured both by the Karl Fischer method and by vacuum drying at 100° over phosphorus pentoxide as previously described (10).

Qualitative tests for metal ion were made on both the saturated aqueous solutions of the chelates and suspensions in dilute hydrochloric acid at a pH of 3.0 after an hour. The two copper chelates gave negative tests in both cases using ferrocyanide and ferricyanide indicators. The ferrous chelate showed positive tests for ferrous ion in both media, as did the cobalt and zinc chelates. In no case was a greater intensity of color produced from the suspensions in dilute hydrochloric acid after an hour than from the aqueous solutions, however. These indications are in agreement with the stability constants of INH-metal chelates determined by Albert (3).

Measurement of Partition Coefficients.—Oleyl alcohol (DuPont Co., b. p. 137–142°/2 mm.) was used to extract the chelates from 10^{-4} to 10^{-3} *M* aqueous solutions as previously described (10). The use of buffers to provide a uniform pH in the aqueous phase was considered inadvisable, since change of pH obviously alters the ligand-metal ratio, and many buffer systems are capable of undergoing salt or complex formation with metal ions or chelates. In the case of the equilibrium determinations, the 1:1 chelate showed sufficient solubility in the aqueous phase to permit determinations to be made at two concentrations. For the 2:1 chelate however, values for only one concentration are reported, since at higher concentrations, the solubility limit in oleyl alcohol was exceeded, and at lower concentrations, the accuracy of measurement by the spectrophotometer used (Beckman Model DU) is questionable.

Determination of Antitubercular Activities.—Mice infected with *M. tuberculosis* H37Rv were fed the chelates as previously reported (7), and the mean survival times were compared with those of control mice receiving either no drug or INH as standard.

[1] A gift of the Massachusetts General Hospital.

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Interaction of Preservatives with Macromolecules I.

Binding of Parahydroxybenzoic Acid Esters by Polyoxyethylene 20 Sorbitan Monooleate (Tween 80)*

By N. K. PATEL and H. B. KOSTENBAUDER

A quantitative evaluation of the degree of intermolecular association between *p*-hydroxybenzoic acid esters and Tween® 80 was obtained by means of an equilibrium dialysis study employing a semipermeable nylon membrane. Data obtained facilitate calculation of the quantity of preservative which must be added to an aqueous system containing a known concentration of Tween® 80 in order to have the desired concentration of unbound preservative.

NUMEROUS REPORTS have appeared in recent years concerning the inactivation of various preservatives in the presence of several nonionic surfactants (1–9) and vegetable gums (10–12) commonly employed in pharmaceutical systems. One of the examples which has most frequently been cited is that of the inhibition of phenolic preservatives in the presence of surfactants which are polyether derivatives of fatty acid esters. Several investigators have suggested that this phenomenon can probably be attributed to the formation of a complex between the preservative and the surfactant (8, 9).

As demonstrated by Allawala and Riegelman (13, 14) in their studies of iodine solubilized by surface-active agents, it is the thermodynamic activity rather than the stoichiometric concentration of the toxic substance which is related to the antimicrobial activity. Therefore, in the presence of an agent which is capable of forming a complex with the preservative, it is to be expected that both the thermodynamic and antimicrobial activity will be diminished. The present investigation was undertaken to obtain a quantitative evaluation of the association between *p*-hydroxybenzoic acid esters and Tween® 80[1], for the purpose of relating this possible interaction to the reported inhibition of the preservative.

Higuchi and Lach (15) and Guttman and Higuchi (16) demonstrated the tendency of phenols to form molecular complexes with polyethers such as the polyethylene glycols. It would thus be expected that phenolic preservatives such as the *p*-hydroxybenzoates might form molecular complexes with the ethereal oxygen of the polyoxyethylene groups of the Tween molecule.

A convenient method for investigating a system of this type is an equilibrium dialysis technique which utilizes a membrane that permits free passage of the *p*-hydroxybenzoate, but is impermeable to the macromolecule. At equilibrium the activity of the *p*-hydroxybenzoate will be identical on both sides of the membrane, and for reasonably dilute solutions it may be assumed that the concentration of free *p*-hydroxybenzoate on both sides of the membrane will be essentially equal. Thus, by placing the macromolecule on one side of the membrane and determining the concentration of *p*-hydroxybenzoate on the opposite side of the membrane, it is possible to determine the concentration of free or unbound preservative in equilibrium with the macromolecule. It is to be expected that the effectiveness of the preservative in the presence of the macromolecule might parallel the concentration of free *p*-hydroxybenzoate. It should perhaps be noted that the dialysis method permits an estimation of the degree of binding should the association be due to the postulated complex formation or to some other mechanism such as preferential solubility of the preservative within a micelle.

EXPERIMENTAL

Reagents.—Recrystallized methyl *p*-hydroxybenzoate,[2] m. p. 125–126°; recrystallized propyl *p*-hydroxybenzoate,[3] m. p. 94–95°; Tween® 80, a commercial sample.

Selection of the Dialysis Membrane.—Bags fashioned from Visking cellulose casing were originally investigated for use as dialysis membranes; but they were found to be unsatisfactory, since they were not impermeable to the Tween.® The

* Received August 9, 1957, from the School of Pharmacy, Temple University, Philadelphia, Pa.
Presented to the Fourth Pan-American Congress of Pharmacy and Biochemistry, Washington, D. C., November, 1957.

[1] Tween® 80 is polyoxyethylene 20 sorbitan monooleate (Atlas Powder Co., Wilmington, Del.).

[2] Methyl Parasept,® Purified, supplied through the courtesy of Heyden Newport Chemical Corporation, New York.

[3] Propyl Parasept®, Purified, supplied through the courtesy of Heyden Newport Chemical Corporation, New York.

presence of Tween® on both sides of the membrane was readily detected both by the foaming tendency of the solution upon agitation, and by the white, oily precipitate formed when a small quantity of the Tween® solution was added to a 5% aqueous solution of phenol.

A thin nylon membrane[4] was found to be highly satisfactory, since it proved to be permeable to the *p*-hydroxybenzoate and impermeable to the macromolecule. In the course of the investigation it was found that the nylon membrane had a tendency to bind the *p*-hydroxybenzoate, but this binding was readily accounted for in the calculations.

Dialysis Method.—The dialysis membrane was employed in the form of small nylon sacks which were made as uniform in size as possible. Into each bag was placed 20 ml. of an aqueous solution of the preservative, and the bag was then tightly closed by knotting the open end. Each nylon bag was then placed in a wide-mouth, ground-glass stoppered bottle containing 20 ml. of the Tween® 80 solution. The bottles were then stoppered tightly and agitated for fifteen hours at 30°. Where very high concentrations of paraben were desired it was necessary to add additional solid paraben to the external solution.

Samples containing only purified water as the external solution were also included to verify attainment of equilibrium and to provide data on the binding of the preservative by the nylon membrane.

After equilibration, samples were removed from the external solution and the concentration of *p*-hydroxybenzoate was determined spectrophotometrically at a wavelength of 255 mμ. It was found that the concentration of *p*-hydroxybenzoate outside the bag could be satisfactorily determined either by a direct spectrophotometric analysis or by calculation after correcting for the amount of paraben bound by the nylon.

Solubility Method.—The solubility of methyl *p*-hydroxybenzoate was studied by placing an excess of the ester (0.25 Gm.) in rubber capped vials containing 10 ml. of Tween® 80 solution. The vials were then agitated for forty-eight hours at 27°, and the concentration of methyl *p*-hydroxybenzoate in solution was determined spectrophotometrically. It was found that the Tween® did not interfere with the spectrophotometric assay. The samples were diluted with water many fold before the spectrophotometric determinations were performed, and the absorption curve for the paraben in these extremely dilute Tween® solutions was identical to that in distilled water.

RESULTS AND DISCUSSION

Binding of Methyl *p*-Hydroxybenzoate by Nylon.—In the course of the investigation it was found that even when the system consisted only of the *p*-hydroxybenzoate, purified water, and the nylon membrane, the total quantity of *p*-hydroxybenzoate found in solution at equilibrium was less than that added to the system. This discrepancy was attributed to binding of the *p*-hydroxybenzoate by the nylon membrane and was found to be a reversible binding which is dependent on both the size of the nylon membrane and the concentration of free paraben. By keeping the size of the dialysis bags constant throughout the experiment, the *p*-hydroxybenzoate bound by the nylon was a function only of the concentration of free *p*-hydroxybenzoate. These data are illustrated in Fig. 1.

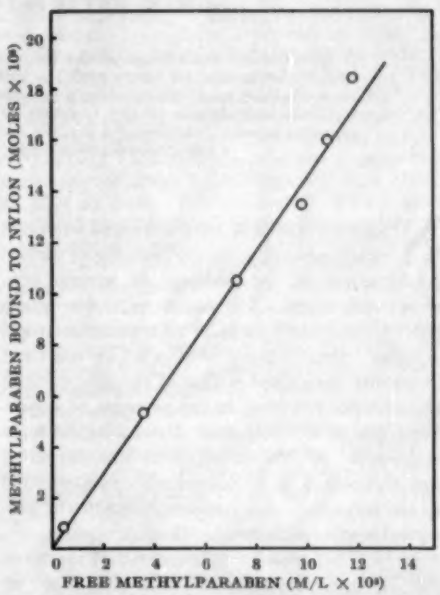

Fig. 1.—Binding of methyl *p*-hydroxybenzoate by the nylon membrane as a function of the concentration of free methyl *p*-hydroxybenzoate in water at 30°.

The binding of the paraben by the nylon is not unusual when the protein-like nature of the nylon is considered. Kostenbauder and Higuchi (17, 18) demonstrated that amides form molecular complexes in aqueous solution with certain organic compounds which act as proton donors, and it is probable that a similar interaction occurs between the phenolic preservative and the amide groups of the nylon.

The possibility of the surfactant being bound by the nylon membrane was not investigated, since it was assumed that any Tween® bound by the nylon would be negligible in comparison to the relatively high concentrations of Tween® added to the system. The validity of this assumption was confirmed by the solubility studies.

Dialysis Study of the Interaction Between Methyl *p*-Hydroxybenzoate and Tween® 80.—The degree of binding of methyl *p*-hydroxybenzoate by Tween® 80 was determined as the difference between the total amount of *p*-hydroxybenzoate added to the system and the *p*-hydroxybenzoate found at equilibrium as free *p*-hydroxybenzoate and *p*-hydroxybenzoate bound to the nylon membrane. The concentration of free *p*-hydroxybenzoate was determined spectrophotometrically in the internal

[4] Supplied through the courtesy of Youngs Rubber Corporation, New York.

solution, and the *p*-hydroxybenzoate bound to the nylon was determined from Fig. 1.

Let P_t = total *p*-hydroxybenzoate added, P_b = *p*-hydroxybenzoate bound to Tween®, P_n = *p*-hydroxybenzoate bound to nylon, P_f = free *p*-hydroxybenzoate inside = free *p*-hydroxybenzoate outside, then $P_b = P_t - (2P_f + P_n)$.

An alternative method is to determine directly the concentration of *p*-hydroxybenzoate in solution outside the bag and the concentration of free *p*-hydroxybenzoate inside the bag. Then the *p*-hydroxybenzoate bound by the Tween® is the difference between the concentration outside and the concentration inside. The two methods were in excellent agreement over the concentration range employed.

Figure 2 shows the binding of methyl *p*-hydroxybenzoate by Tween® 80 as a function of both Tween® 80 concentration and free methyl *p*-hydroxybenzoate concentration. Figure 3 indicates

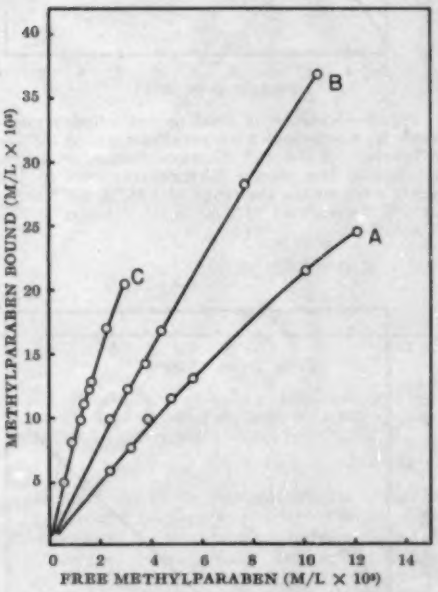

Fig. 2.—Binding of methyl *p*-hydroxybenzoate by Tween® 80 at 30°. A—3% (W/V) Tween® 80; B—5% (W/V) Tween® 80; C—10% (W/V) Tween® 80.

that the ratio, *r*, of total *p*-hydroxybenzoate in solution to the concentration of free *p*-hydroxybenzoate is a function of the concentration of Tween® 80. It is evident from these data that there is considerable interaction occurring between the preservative and the surfactant. At a concentration of 5% Tween® 80 only 22% of the total paraben present exists as free paraben, and at a concentration of 10% Tween® 80 only 12% of the total paraben exists as free paraben. The data illustrated in Fig. 3 facilitate the determination of the quantity of preservative which must be added to a system containing a known concentration of Tween® 80 in order to have the desired concentration of free preservative. Multiplying the desired concentration of free methyl *p*-hydroxybenzoate by the appropriate *r* value gives the concentration of total methyl *p*-hydroxybenzoate which must be employed.

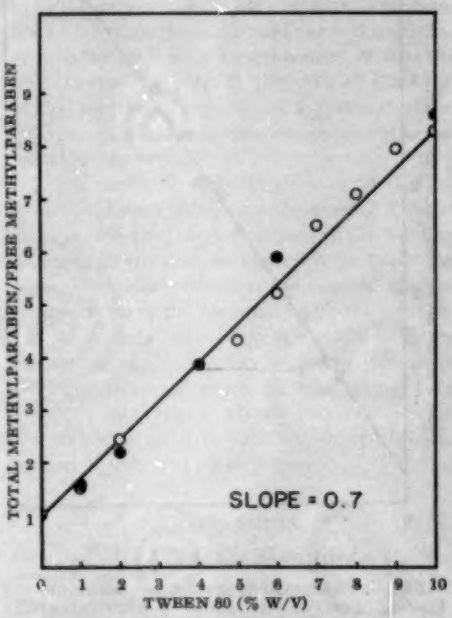

Fig. 3.—A plot showing the ratio, *r*, of total methyl *p*-hydroxybenzoate to free methyl *p*-hydroxybenzoate, at 30°, in aqueous solutions containing varying concentrations of Tween® 80. ○—Concentration of methyl *p*-hydroxybenzoate on each side of the membrane would be 3.84×10^{-3} molar in absence of Tween® 80; ●—concentration on each side of membrane would be 6.24×10^{-3} molar in absence of Tween® 80.

Figure 4 is a Langmuir-type plot of the binding of methyl *p*-hydroxybenzoate by three and five % solutions of Tween® 80. The data are plotted in this manner to obtain the limiting binding capacity of the Tweens unit at infinitely high methyl *p*-hydroxybenzoate concentration. The *Y*-intercept is the reciprocal of the limiting quantity of methyl *p*-hydroxybenzoate bound per gram of Tween® 80. This graph is of particular interest in the present investigation, since the existence of the *Y*-intercept suggests that complex formation is a factor in the binding of the preservative by the macromolecule. If the sole interaction in the system had been a partitioning of the preservative into a micelle, the expected curve would be a straight line through the origin.

The *Y*-intercept in Fig. 4 indicates that the limit-

ing binding capacity of the Tween® 80 is approximately 0.004 moles of methyl *p*-hydroxybenzoate per gram of Tween® 80. Using the method described by Schönfeldt (19, 20), and employing Polyethylene Glycol 4000[8] and Polyethylene Glycol 6000[8] as standards, the polyoxyethylene content of

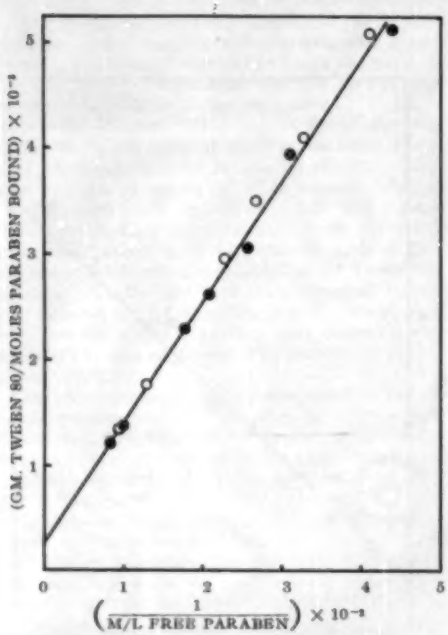

Fig. 4.—Langmuir-type plot to determine the limiting quantity of methyl *p*-hydroxybenzoate bound by Tween® 80 in infinitely high methyl *p*-hydroxybenzoate concentration. ●—3% (W/V) Tween® 80; ○—5% (W/V) Tween® 80.

the Tween® 80 was approximated as 0.016 polyoxyethylene groups per gram of Tween® 80. These results would correspond to a limiting ratio of 1 mole of bound methyl *p*-hydroxybenzoate for each 4 polyoxyethylene units of the Tween® 80. All data were obtained using a single lot of Tween® 80.

Dialysis Study of the Interaction between Propyl *p*-Hydroxybenzoate and Tween® 80.—Figure 5 shows the binding of propyl *p*-hydroxybenzoate as a function of Tween® 80 concentration. The interaction of the propyl ester with nylon was not studied; therefore, the degree of binding by the Tween® was determined by direct analysis of the solution on both sides of the membrane. Comparison of Figs. 3 and 5 indicates that propyl *p*-hydroxybenzoate has greater affinity for Tween® 80 than has the methyl ester. At a concentration of 5% Tween® 80 only 4.5% of the total propyl *p*-hydroxybenzoate was found to exist in the unbound form.

Solubility Study.—Figure 6 illustrates the solubility of methyl *p*-hydroxybenzoate as a function of

[8] Carbowax® 4000 and Carbowax® 6000, respectively, Union Carbide Chemicals Co., New York.

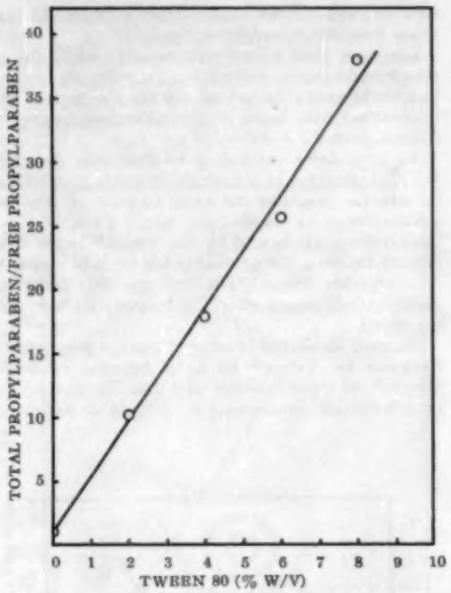

Fig. 5.—Ratio, *r*, of total propyl *p*-hydroxybenzoate to free propyl *p*-hydroxybenzoate, at 30°, as a function of Tween® 80 concentration. Concentrations of free propyl *p*-hydroxybenzoate in this study were within the range of 1.58×10^{-3} molar at 2% Tween® 80 to 0.50×10^{-3} molar at 8% Tween® 80.

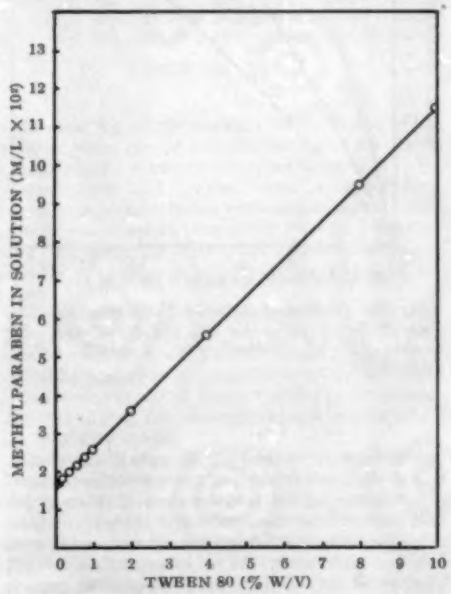

Fig. 6.—Solubility of methyl *p*-hydroxybenzoate in water at 27° as a function of Tween® 80 concentration.

Tween® 80 concentration. The solubility determinations were made at a temperature of 27°, since at 30° a saturated solution of methyl *p*-hydroxybenzoate in some concentrations of Tween® 80 resulted in the formation of a white, oily precipitate (21) which was not readily filterable.

Figure 7 was obtained by treating the solubility data in the same manner as the dialysis data. In the solubility study, the free *p*-hydroxybenzoate concentration was a constant corresponding to the

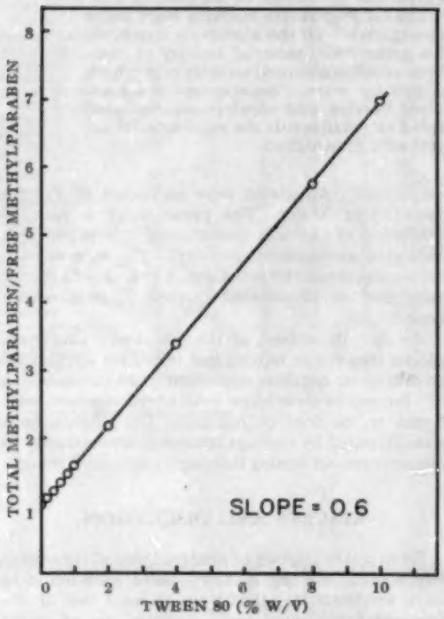

Fig. 7.—Ratio, *r*, of total methyl *p*-hydroxybenzoate to free methyl *p*-hydroxybenzoate obtained from the solubility study illustrated in Fig. 6.

water solubility of the paraben; and the concentration of bound paraben was obtained as the difference between the total paraben in solution and the free paraben concentration. Comparison of Figs. 3 and 7 indicates that the values obtained for the slope in the solubility and dialysis studies are in fairly good agreement. This suggests that the solubilization of the methyl *p*-hydroxybenzoate and the binding as determined in the dialysis studies can probably be attributed to the same mechanism. This agreement between the solubility study and the dialysis study also supports the validity of the assumption that any binding of Tween® by the nylon membrane can be neglected in the dialysis studies.

SUMMARY

1. The interaction of the methyl and propyl esters of *p*-hydroxybenzoic acid with Tween® 80 has been studied quantitatively by means of a dialysis method employing a semipermeable nylon membrane.

2. A relatively high degree of interaction has been observed, and the binding has been found to be a function of both the concentration of unbound *p*-hydroxybenzoate and the concentration of Tween® 80. At a concentration of five per cent Tween® 80 only 22 per cent of the total methyl *p*-hydroxybenzoate and 4.5 per cent of the total propyl *p*-hydroxybenzoate are present as unbound preservative.

3. A study of the solubility of methyl *p*-hydroxybenzoate in aqueous solutions of Tween® 80 indicated that the mechanism of the binding observed in the dialysis studies is probably the same mechanism which is responsible for the solubilization of the preservative by the Tween®.

4. The data obtained in this study facilitate calculation of the quantity of methyl or propyl *p*-hydroxybenzoate which must be added to a system containing a known concentration of Tween® 80 in order to have the desired concentration of unbound preservative.

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The *In Vitro* Antibacterial Activity of Essential Oils and Oil Combinations*

By JASPER C. MARUZZELLA and PERCIVAL A. HENRY

The *in vitro* antibacterial activity of thirty-five volatile oils, five fixed oils, five infused oils, and ninety-five combinations of these oils was ascertained using the filter paper disk method. The oils and oil combinations were tested against five bacteria. Eucalyptus, cinnamon, and origanum red exhibited the greatest antibacterial activity while the volatile oils of cedar wood and myrrh as well as all of the fixed and infused oils were found to possess no activity. The Gram-positive bacteria were more susceptible to the volatile oils than the Gram-negative. Of the ninety-six combinations of oils only three were found to exhibit greater antibacterial activity as compared with each oil used separately. The three combinations of volatile oils which showed this enhancement of antibacterial activity were: eucalyptus-cinnamon-dwarf pine needle, eucalyptus-cinnamon-juniper berries, and eucalyptus-cinnamon-niaouli. When fixed or infused oils were added to volatile oils the antibacterial activity of the latter was markedly diminished.

THE MAJORITY of the testing for the antibacterial action of oils and their constituents has been performed with the well-known phenol coefficient method (1–11). Using this method it was found that emulsified mixtures of two or more oils increased the bactericidal efficiency as compared with each principle used separately (12). Recently the filter paper disk method has been employed to measure the antibacterial activity of oils (13, 14). The present investigation is concerned with the *in vitro* antibacterial activity of oils with special consideration to mixtures of two or more oils in order to ascertain whether antibacterial activity would be enhanced or diminished.

MATERIALS AND METHODS

The detection of the antibacterial activity of various volatile oils, fixed oils, and infused oils in addition to mixtures of two or more volatile oils, volatile and fixed oils, and volatile and infused oils was studied on growing cultures of five organisms. The organisms used were: *Salmonella typhosa, Micrococcus citreus, Proteus morgani, Bacillus brevis,* and *Micrococcus pyogenes var. albus.* Thirty-six hour cultures of these organisms were grown in nutrient broth (Difco). Stock cultures were maintained on fresh nutrient agar slants and subcultured every seven days.

The method used for determining the presence or absence of antibacterial activity was a slight modification of Vincent's (15) qualitative filter paper disk diffusion plate method. In this method small sterile disks (12.5 mm. diameter) of filter paper were thoroughly saturated with the oil or oil mixture and then placed on nutrient agar (Difco) plates which had been previously seeded with 2 cc. of a thirty-six hour nutrient broth culture of the organism. All dishes were conducted in triplicate with two or three disks per dish. The measurement of each zone of inhibition represents a mean value of a minimum of six recordings. All dishes were incubated at 37° for twenty-four hours. The presence of a zone of inhibition of any size surrounding the paper disk indicated antibacterial activity. The zone of inhibition was measured to 0.5 mm. by means of a metric ruler and an illuminated Quebec bacteria colony counter.

As the oils arrived at the laboratory they were placed into sterile bottles and tested for sterility by streaking on nutrient agar slants and incubated at 37° for one to three days. All of the oils used were found to be free of bacteria. Oil combinations were prepared by adding the appropriate quantity to one another and mixing thoroughly in a sterile bottle.

RESULTS AND DISCUSSION

From Table I it may be observed that of the thirty-five volatile oils tested, thirty-three were found to have antibacterial activity on at least one of the five organisms employed. Volatile oils of cedar wood and myrrh were found to have no activity. The five fixed oils, castor, cod liver, vitamin K_1, olive, and white mineral, and the five infused oils, asafetida, burdock, henbane, lobelia, and mullein, were found to possess no antibacterial activity. The effect of the volatile oils against each organism was found to be of the following order (figures indicate the sum of the zones of inhibition caused by the oils): *M. citreus,* 174 mm.; *B. brevis,* 168 mm.; *Micrococcus pyogenes var. albus,* 102 mm.; *P. morgani,* 63 mm.; and *S. typhosa,* 48 mm. Therefore the volatile oils are more effective against Gram-positive bacteria than against Gram-negative. This is in agreement with the work of Turkheim (13) and Maruzzella and Lichtenstein (14) who likewise found volatile oils to be more effective against Gram-positive bacteria. Furthermore the latter investigators also obtained negative results with fixed and infused oils on bacteria. It may also be observed from Table I that the volatile oils showing the greatest sum total of zones of inhibition were: eucalyptus, cinnamon, and origanum red.

Table II shows the results obtained on bacteria with 47 combinations of two volatile oils (1:1), 15 combinations of three volatile oils (1:1:1), 28 combinations of volatile and fixed oils (1:1), and five combinations of volatile and infused oils (1:1). With

* Received June 1, 1957, from Long Island University, Biology Department, Brooklyn, N. Y.

TABLE I.—INHIBITORY ACTIVITY OF OILS

Volatile Oils	Zone of Inhibition, mm.[a] S. typhosa	M. citreus	P. morganii	B. brevis	M. albus	Sum[b]
Amber, Rect.	0[c]	2	0	2	2	6
Anise	0	3	0	4	2	9
Bay	0	7	3	3	3	16
Bergamot	4	7	0	6	3	20
Cajuput	0	10	0	11	5	26
Caraway	0	10	0	5	3	18
Cedar Wood	0	0	0	0	0	0
Chenopodium	0	5	3	3	3	14
Cinnamon	0	11	8	12	10	41
Clove	0	3	0	3	2	8
Cubeb	0	0	0	2	0	2
Dwarf Pine Needle	3	3	5	3	3	17
Eucalyptus	5	10	10	12	8	45
Geranium Algerian	2	12	2	5	2	23
Hemlock	1	0	2	0	0	3
Juniper Berries, Rect. Dutch	5	2	9	2	3	21
Juniper Tar	0	10	0	2	3	15
Lemon	0	0	0	13	0	13
Myrrh	0	0	0	0	0	0
Neroli	5	3	4	10	3	25
Niaouli	0	10	6	10	3	29
Origanum Red	0	15	0	15	10	40
Peppermint	5	0	0	5	2	12
Petitgrain, Paraguay	3	0	3	4	2	12
Pimento	0	10	0	4	3	17
Pinus Sylvestris	8	5	0	6	5	24
Rectified Tar	0	10	0	0	6	16
Rosemary	5	7	0	5	6	23
Sandalwood	0	3	0	2	2	7
Sassafras	0	3	0	3	2	8
Spike Lavender	0	5	0	5	3	13
Sweet Birch	0	0	0	2	0	2
Sweet Orange	0	0	0	2	0	2
Turpentine	2	3	8	4	3	20
Wintergreen	0	5	0	3	0	8

[a] Measurement from disk edge to zone edge.
[b] Sum of zones of inhibition for each oil.
[c] Zone of inhibition absent.

TABLE II.—INHIBITORY ACTIVITY OF COMBINATIONS OF OILS

Oil Combinations	Zone of Inhibition, mm.[a] S. typhosa	M. citreus	P. morganii	B. brevis	M. albus	Sum[b]
Volatile (1:1)						
Anise-Clove	0[c]	3	0	4	2	9
Anise-Hemlock	0	3	0	4	2	9
Chenopodium-Cedar Wood	0	10	0	14	7	31
Cinnamon-Clove	0	10	0	10	10	30
Cinnamon-Cubeb	0	10	0	13	10	33
Cinnamon-Dwarf Pine Needle	0	15	0	15	5	35
Cinnamon-Juniper Berries	0	10	0	15	5	30
Cinnamon-Niaouli	0	10	0	11	10	31
Cinnamon-Pinus Sylvestris	0	10	0	12	3	25
Cinnamon-Sandalwood	0	10	0	12	5	27
Cinnamon-Sweet Birch	0	7	0	10	3	20
Cinnamon-Turpentine	0	10	0	15	14	39
Cubeb-Cedar Wood	0	0	0	1	0	1
Cubeb-Dwarf Pine Needle	3	2	4	3	2	14
Cubeb-Juniper Berries	3	2	5	2	3	15
Cubeb-Pinus Sylvestris	2	3	0	2	1	8
Dwarf Pine Needle-Cedar Wood	3	2	4	2	2	13
Eucalyptus-Anise	0	5	0	5	5	15
Eucalyptus-Cajuput	0	4	0	12	5	21
Eucalyptus-Cedar Wood	3	5	0	5	5	18
Eucalpytus-Chenopodium	0	9	0	8	6	23

TABLE II (*Continued*)

Oil Combinations	Zone of Inhibition, mm.[a] S. typhosa	M. citreus	P. morganii	B. brevis	M. albus	Sum[b]
Eucalyptus-Cinnamon	0	12	0	15	15	42
Eucalyptus-Clove	0	5	0	5	5	15
Eucalyptus-Cubeb	5	5	4	5	6	25
Eucalyptus-Dwarf Pine Needle	8	10	0	8	6	32
Eucalyptus-Juniper Berries	8	9	0	10	5	32
Eucalyptus-Myrrh	3	5	0	5	3	16
Eucalyptus-Niaouli	0	8	0	12	5	25
Eucalyptus-Pinus Sylvestris	7	10	0	15	3	35
Eucalyptus-Rosemary	9	8	0	10	9	36
Eucalpytus-Sandalwood	3	5	5	4	5	22
Eucalyptus-Sweet Orange	7	5	0	6	4	22
Eucalyptus-Wintergreen	0	5	0	5	5	15
Juniper Berries-Cedar Wood	2	0	3	2	2	9
Juniper Berries-Dwarf Pine Needle	2	2	7	2	2	15
Niaouli-Clove	0	5	0	4	3	12
Niaouli-Cubeb	0	2	3	3	1	9
Niaouli-Pinus Sylvestris	0	8	0	12	7	27
Niaouli-Rectified Tar	0	6	0	6	4	16
Niaouli-Sweet Birch	0	4	0	4	3	11
Niaouli-Wintergreen	0	3	0	4	3	10
Peppermint-Pimento	0	4	0	3	3	10
Turpentine-Clove	3	5	0	4	4	16
Turpentine-Hemlock	0	0	4	2	3	9
Turpentine-Juniper Berries	3	2	9	2	3	19
Turpentine-Origanum Red	0	10	0	0	8	18
Turpentine-Wintergreen	0	5	0	4	3	12
Volatile (1:1:1)						
Cedar Wood-Cubeb-Bay	0	3	2	3	2	10
Eucalyptus-Chenopodium-Anise	0	5	0	3	4	12
Eucalyptus-Chenopodium-Cedar Wood	4	6	0	4	5	19
Eucalyptus-Chenopodium-Hemlock	5	6	0	5	4	20
Eucalyptus-Chenopodium-Peppermint	3	5	0	4	3	15
Eucalyptus-Cinnamon-Clove	0	4	3	4	3	14
Eucalyptus-Cinnamon-Dwarf Pine Needle	8	11	0	15	18	51
Eucalyptus-Cinnamon-Juniper Berries	10	12	0	17	10	58
Eucalyptus-Cinnamon-Niaouli	7	14	0	15	15	51
Eucalyptus-Cinnamon-Rosemary	0	5	3	6	4	18
Sassafras-Origanum Red-Chenopodium	0	5	3	3	3	14
Turpentine-Geranium Algerian-Rosemary	0	5	3	6	5	19
Turpentine-Neroli-Geranium Algerian	0	2	2	3	5	12

TABLE II (*Continued*)

Oil Combinations	Zone of Inhibition, mm.[a] *S. typhosa*	*M. citreus*	*P. morganii*	*B. brevis*	*M. albus*	Sum[b]
Wintergreen-Sweet Birch-Lemon	0	2	0	2	0	4
Wintergreen-Sweet Orange-Sassafras	0	4	0	3	0	7
Volatile and Fixed (1:1)						
Castor-Cedar Wood	0	0	0	0	0	0
Castor-Eucalyptus	0	5	0	4	5	14
Castor-Origanum Red	0	2	0	3	0	5
Castor-Turpentine	0	0	0	1	0	1
Cod Liver-Cedar Wood	0	0	0	0	0	0
Cod Liver-Eucalyptus	0	5	0	4	7	16
Cod Liver-Origanum Red	0	3	0	4	3	10
Cod Liver-Turpentine	0	0	0	2	2	4
Olive-Amber, Rect.	0	0	0	2	0	2
Olive-Bay	0	2	0	3	3	8
Olive-Bergamot	0	1	0	2	2	5
Olive-Cajuput	0	5	0	4	5	14
Olive-Caraway	0	0	0	2	0	2
Olive-Cedar Wood	0	0	0	0	0	0
Olive-Chenopodium	0	2	0	3	0	5
Olive-Clove	0	3	0	2	2	7
Olive-Eucalyptus	0	5	0	4	8	17
Olive-Juniper Tar	0	2	0	2	2	6
Olive-Origanum Red	0	4	0	5	2	11
Olive-Peppermint	0	0	2	2	0	4
Olive-Sassafras	0	0	0	1	0	1
Olive-Turpentine	0	2	4	1	0	7
Olive-Wintergreen	0	0	0	0	0	0
Vitamin K$_1$-Eucalyptus	0	7	10	4	6	27
White Mineral-Cedar Wood	0	0	0	0	0	0
White Mineral-Eucalyptus	0	7	0	5	7	19
White Mineral-Origanum Red	0	7	0	8	0	15
White Mineral-Turpentine	0	2	6	2	3	13
Volatile and Infused (1:1)						
Asafetida-Eucalyptus	0	3	3	3	5	14
Burdock-Eucalyptus	0	3	5	2	5	15
Henbane-Eucalyptus	0	6	5	5	5	21
Lobelia-Eucalyptus	0	5	7	5	5	22
Mullein-Eucalyptus	0	6	3	3	5	17

[a] Measurement from disk edge to zone edge.
[b] Sum of zones of inhibition for each oil.
[c] Zone of inhibition absent.

the combinations of two volatile oils none were found to enhance antibacterial activity. Two combinations, anise-clove and anise-hemlock, were found to have the same effect as anise used alone. The remaining 45 mixtures of two volatile oils showed a decrease in antibacterial activity as compared to each oil used separately. With the combinations of three volatile oils the following mixtures were found to exhibit greater antibacterial activity as compared to each oil used separately (compare Tables I and II): eucalyptus-cinnamon-dwarf pine needle, eucalyptus-cinnamon-juniper berries, and eucalyptus-cinnamon-niaouli. The remaining combinations of three volatile oils were found to produce a decrease in antibacterial activity. All mixtures of volatile with fixed oils and volatile with infused oils resulted in a marked decrease in the antibacterial activity of the volatile oil.

The mechanism of action of the antibacterial activity of volatile oils is not known but some clues are given by the work of Gal'perin and Dunaeva (16, 17). These investigators found that many volatile oils produced arresting effects on dehydrogenases of paramecia and helminths. This may also be true of bacteria.

The reason why most oil combinations result in a decrease in antibacterial activity is likewise unknown. Perhaps a loss of power of diffusion would explain this phenomenon. Indeed this would seem to be true for mixtures of volatile oils with fixed and infused oils. Furthermore it would not be unreasonable to assume that fixed and infused oils might coat the active constituents of the volatile oils rendering them inactive to the bacterial enzymes. In the work of Miller (12) mixtures of volatile oils were found to enhance bactericidal power by killing the organisms in a shorter period of time. With the method used in this investigation enhancement of antibacterial power was indicated by an increase in the size of the zone of inhibition produced at a fixed period of time. With these different methods it is possible to obtain divergent results.

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Comparative Dissolution Rates of Weak Acids and Their Sodium Salts*

By EINO NELSON

The dissolution rates of several weak acids and their sodium salts were studied in mediums representing gastrointestinal fluids with respect to pH. Marked differences existed between dissolution rates of acids and their sodium salts; the sodium salts always dissolved much more rapidly than the free acid. The experimental method was based on solution by free convection and an expression was derived to express dissolution rate from a circular plate suspended vertically in dissolution medium as a function of the variables involved. The validity of the expression was shown for sodium chloride dissolving in water. The significance of the results in acid-salt rate studies to oral administration of drugs was discussed.

It was shown in some recent work that marked differences existed in the rate with which several amine and alkanolamine salts of the weak acid theophylline dissolved (1). It was suggested that these differences were related to theophylline blood levels after oral administration of the salts in clinical studies. It was also shown that all salts dissolved more rapidly than the free acid in a pH range representing those likely to be encountered by drug after oral administration. In this pH range, the rates of solution of both the free acid and the salts were relatively independent of medium.

The differences in rate observed between the salts and between any salt and the free acid were attributed to the properties of the diffusion layer which surrounds a dissolving solid. The pH of the diffusion layer surrounding a salt of a weak acid, if the salt is a highly water-soluble compound will be relatively independent of dissolution medium pH and this property will exert a strong influence on rate of solution. Generally speaking, rate will increase with diffusion layer pH since concentration increases with this quantity but other factors are also involved.

In view of the above, a marked difference should exist in the rate with which the sodium and potassium salts of weak acids dissolve in comparison to the free acids. The work now reported was conducted to determine the comparative solution rates for several weak acids and their sodium salts in mediums representing gastrointestinal fluids with respect to pH. Another purpose of the work was to develop a technique for studying dissolution rate without requiring an assay method for the dissolving specie.

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EXPERIMENTAL

Theory of the Method.—Dissolution rate was studied by a method in which free convection, diffusion coefficient and concentration in the diffusion layer were the rate determining factors. An expression to relate average rate per unit area from a two dimensional rectangular surface, suspended vertically in dissolution medium, with the several parameters involved in the process has been previously

$$R'_m = 0.726\, DC_s \left(\frac{g}{HD\nu}\frac{\Delta\rho}{\rho_0}\right)^{1/4} \quad \text{(Eq. 1)}$$

described (2). This expression, describes mean rate per unit area in terms of diffusion coefficient D, concentration in the diffusion layer Cs, height of the rectangular plate H, viscosity of the diffusion layer ν, difference in density between the diffusion layer and the medium $\Delta\rho$, gravitational constant g and density of the medium ρ_0 (all terms in cgs units). The correctness of this expression to describe rate of dissolution of thin plates of sodium chloride in water was established (2).

The test specimens used in the present work were thin compressed circular pellets of several weak acids and their sodium salts mounted in a manner such as to allow vertical suspension in dissolution medium. The dissolution rate observed from a circular surface should also be a mean rate since rate varies with height of infinitesimally thin vertical sections across the surface. This mean rate should be expressible by the mean value of equation 1 after relating the quantity H to an appropriate variable. The quantity H may be expressed as a function of r and θ, where r is the radius of the circular surface; the quantity θ is the angle between a horizontal line through the center of the surface and any given radius forming the third side of a right triangle bounded by these two lines and a vertical line whose length is one-half the length of a corresponding infinitesimally thin vertical section. Expressing H in terms of r and θ the mean value of equation 1 will be given by evaluation of the following expression:

$$R_m = \int_{\pi/180}^{\pi/2} \frac{0.462 DC_s}{\sin^{1/4}\theta}\left(\frac{g}{2r D\nu}\frac{\Delta\rho}{\rho_0}\right)^{1/4} d\theta \quad \text{(Eq. 2)}$$

In equation 2 the lower limit must be taken at some small angle since the integral has no limit at $\theta = 0$. This is an academic consideration only and equation 2 may be integrated graphically to give

$$R_m = 0.884 D C_s \left(\frac{g}{2\nu D} \frac{\Delta\rho}{\rho_s}\right)^{1/3} \quad \text{(Eq. 3)}$$

It is seen from equation 3 that dissolution rate observed from a circular surface will be constant at constant temperature and surface area. It is implicitly understood that concentration in the medium is always small with respect to the concentration of the diffusion layer.

Preparation of Test Samples.—The test pellets were prepared from powdered samples of the weak acids and their sodium salts by compressing in a $^1/_2$-in. die with flat-faced punches at 50,000 psi. The compressions were made in a Carver press modified to allow compression using standard tablet machine punches and dies. The die wall and punch faces were lubricated with a slurry of magnesium stearate in alcohol prior to compressions. All compounds were of USP, NF or better quality and used without further purification. The compounds used are listed in Table I. Test pellets of sodium chloride used to check the validity of equation 3 were prepared from −200 mesh analytical reagent grade chemical by compression in vacuum by a technique previously described (1). These pellets were 1.3 cm. in diameter. In the case of weak acids and their sodium salts it was sometimes necessary to precompress the powders before intact pellets could be formed.

The pellets prepared as described above were mounted flat on ceresin wax coated aluminum strips 2 cm. wide by 4 cm. long.

The apparent density of all pellets was determined by micrometer measurements and weighings.

Test Procedure for Sodium Salts.—The mounted pellets were suspended by means of the nylon thread to the suspension hook of a Sartorius Selecta balance in such a manner as to allow immersion of the entire mounting plate with specimen in 175 ml. dissolution medium positioned over the balance pan. The weight loss was followed directly on the illuminated 0 to 100 mg. scale. No weight changes were recorded until all rotational movement of the test pellet and its mounting had ceased. After equilibrium was established, the times required for apparent losses of 4 or 5 mg. were noted with a stop watch. Readings were recorded only during the dissolution of the first 30 to 40 mg. since a wax wall was left around the undissolved part of the pellets. The presence of this wall was considered to cause a change in the flow pattern across the surface of the specimens. The apparent losses of weight multiplied by the ratios of the densities of the pellets to the difference between the densities of the pellets and mediums gave the amounts dissolved.

The calculations involved in expressing the observed weight losses to the rate units of Table I are illustrated by the following: On the average, an apparent weight loss of 0.0972 mg. per sec. was observed for sodium benzoate dissolving in 0.1 *M* borate buffer. The density of 0.1 *M* borate buffer at 25° was 1.018 and the apparent density of the sodium benzoate pellets was 1.426. Multiplying the apparent loss by the ratio calculated from these densities as described gave an actual loss of 0.3396 mg. per sec. This quantity divided by the area of the pellet, 1.275 sq. cm. gave a rate per unit area of 0.2661 mg./sec.-cm.^{2} This quantity multiplied by 6000 to convert to hundreds of minutes gave the quantity listed in Table I.

TABLE I.—DISSOLUTION RATE OF WEAK ACIDS AND THEIR SODIUM SALTS IN VARIOUS BUFFERS[a,b]

Compound	0.1 *N* HCl pH = 1.5	0.1 *M* Phosphate pH = 6.83	0.1 *M* Borate pH = 9.00	Solubility of free acid Gm/L., 25°	pKa, 25°
Benzoic acid	2.1	14	28	2.9[c]	4.2[c]
Sodium salt	980	1770	1600		
Phenobarbital	0.24	1.2	22	1.0[c]	7.4[f]
Sodium salt	~200	820	1430		
Salicylic acid	1.7	27	53	2.2[c]	2.98[c]
Sodium salt	1870	2500	2420		
Succinic acid	2100	310	310	~77[c]	4.18[d,e]
Sodium salt	6000	3360	2920		
Sulfathiazole	<0.1	~0.50	8.5	0.6[g]	7.26[g]
Sodium salt	550	810	1300		

[a] Rate = mg./100 minutes-cm.^{2} [b] Solution process by free convection. [c] From reference 3. [d] From Reference 4. [e] First hydrogen. [f] From Reference 5. [g] From Reference 6.

Each strip had a small hole punched near the top edge which allowed suspension by means of nylon sewing thread. Pellet mounting was by means of the coating wax and additional molten wax was used to cover the peripheral surface of the pellets thus leaving only one circular face exposed. Before use in tests, the pellets were carefully scraped with a razor blade to remove the excess wax from their edges and lubricant from their faces.

The surface of the pellets were observed during tests to detect the formation of air bubbles since their presence would indicate permeation of solvent into the pellets' interstices.

The test mediums were brought to 25° for tests and since the tests could be made rapidly the change in temperature during tests did not exceed 0.5°. The dissolution rate of sodium chloride was determined at 16° in water.

Procedure for Acids.—With the exception of succinic acid, whose dissolution rate was studied by the same method as the sodium salts, all acids required immersion in dissolution medium maintained at 25° by means of a constant temperature bath. The mounted samples were weighed in air after equilibration with the atmosphere for several days. They were then immersed in dissolution medium for periods up to twenty-four hours, removed, rinsed with distilled water, and left to dry again in the atmosphere. Essentially constant weight was reached in twenty-four hours drying by this method. This method was necessary since it was not practical to continuously observe the pellets for permeation of solvent into their interstices. The loss of weight was recorded and expressed in terms of the appropriate rate units.

RESULTS AND DISCUSSION

Test of Equation 3.—The calculated value for the dissolution rate of sodium chloride at 16°, using the values for the parameters used to check the validity of equation 1 previously (2), was 30.4 mg./min.-cm.^{2} Three determinations with compressed disks of sodium chloride gave values of 29.8, 30.6, and 30.1 mg./min.-cm.^{2} respectively.

Dissolution Rate of the Acids and Salts.—The rate found for the materials studied are listed in Table I along with other information of interest. Determinations were reproducible to within 10% of the mean of three or four determinations. Observation of the pellets during and after tests gave no indication that disintegration occurred.

It is strikingly apparent from examination of Table I that large differences existed between the solution rates of all acids and their sodium salts. Further, the difference existed when the free acid was a relatively water-soluble compound as was the case with succinic acid. The increase in rate of dissolution of the poorly soluble free acids with increasing pH of dissolution medium was expected when their dissociation constants were large enough to allow for the formation of significant quantities of anion in the diffusion layers—particularly in the borate buffer.

A decrease in rate of solution of the salts could be expected with increasing buffer capacity of the dissolution medium. Increasing buffer capacity of the medium would tend to overcome the buffer capacity of the salt in the diffusion layer. Conversely, an increase in rate of solution of the free acids could be expected with an increase in buffer capacity of alkaline dissolution mediums as was the case with theophylline (1).

The absolute difference in rates between acids and their salts was somewhat dependent on the solution process. This is due to the fact that flow rate across the face of the pellets varied in accordance with the density difference term in equation 3. Hence, the differences in rates observed here were greater than those which would be observed were solution taking place under conditions where the Noyes-Whitney law described the solution rate process.

When the salts of poorly soluble weak acids dissolved in mediums that had an insignificant capacity for the anion and small capacity for the free acid, e. g., 0.1 *N* HCl, precipitation of free acid necessarily occurred after the diffusion layer mixed with solvent. The significance of this occurrence when considering the oral administration of therapeutically active weak acids as sodium salts is in the increase in surface area of administered material obtained in the stomach. This increase in surface would increase rate of solution and consequently cause an increase in absorption rate. This consideration was no doubt a factor in explaining the higher and the more rapidly reached maximum blood levels obtained from the administration sodium aspirin in solution as compared to aspirin in tablets (7).

Comments on the Significance of Solution Rate in Absorption of Drugs.—It is appropriate to mention that an understanding and knowledge of the rate of solution of drugs administered in solid form is of importance. In general, availability for absorption is completely dependent on drug being in solution at absorption sites and the rate of the solution step may markedly influence the rate of build-up and maximum level of drug reached in the fluids of distribution.

Absorption of drugs is commonly related to their solubility by many investigators in fields where the subject is pertinent. Relating absorption to solubility is not correct. Solubility refers to the maximum amount of material that can be dissolved in a given volume of solvent at constant temperature in any time long enough to allow attainment of saturation. The absorption of drugs is a dynamic process and ultimate solubility of a drug in fluid at absorption sites is of limited consequence since absorption prevents the attainment of saturated solutions. Solubility of a drug and absorption are related only to the extent that solubility affects solution rate. Solution rate influences absorption rate since it is a preceding process. In addition to solubility, solution rate is dependent on surface, diffusion coefficient and other properties of the dissolving species and the solution process. The condition that exists in the diffusion layer of the salts of weak acids as shown in the work of this paper is another consideration. It should be clear, then, that absorption is properly related to solution rate, not solubility.

SUMMARY

The dissolution rate of the sodium salts of weak acids is much more rapid than the free acids. This is true in mediums possessing hydrogen ion concentrations in the range of those encountered by drugs administered orally. The significance of solution rate in the administration of drugs was discussed.

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Dissolution Rate of Mixtures of Weak Acids and Tribasic Sodium Phosphate*

By EINO NELSON

The dissolution rates of mixtures of several weak acids and trisodium phosphate were determined in stirred mediums and by a process whereby dissolution took place by free convection. The test samples were pelleted mixes of weak acid and trisodium phosphate in a range of mole fraction ratios. The admixture of tribasic sodium phosphate increased the rate of solution of all acids studied and a characteristic relationship was found between rate and fraction of acid in mixture. In all cases, a maximum occurred in plots of rate *vs.* fraction and its location with respect to amount of acid in a mix appeared to be related to the strength of the acid. The maximum rates obtained for the mixes were lower than those obtained from salts of the acids.

THE KINETICS of the solution process have been thoroughly investigated for single substances since the first studies of Noyes and Whitney (1). Apparently no studies have been made when the substance dissolving consists of two or more components. The latter studies are of pharmaceutical interest since many such systems are commonly encountered in dosage forms, e. g., active ingredient plus binder and other materials in tablets or tablet granules and salts of weak acids mixed with buffer salts such as benzylpenicillin sodium with sodium citrate.

Another two-component system of pharmaceutical convenience would be one composed of weak acid plus a strongly basic reacting salt. A rational use of the mixtures would lie in providing an environment near the dissolving mixtures in which the weak acids would be more soluble than the medium in which solution was taking place. In most cases, this condition would exist in or close by the diffusion or interfacial layer between the dissolving solid and medium. This same effect may be obtained in the case of poorly soluble weak acids by use of salts of the acids made with highly water-soluble bases as has been previously shown (2), but some factors may make use of the salts themselves undesirable.

The work now reported was performed to determine the degree of enhancement in dissolution rate of poorly soluble weak acids obtainable by making homogeneous mixtures of these substances and tribasic sodium phosphate. It was also of interest to compare these rates to those obtained when salts of the acids were studied.

EXPERIMENTAL

Materials and Equipment.—The dissolution assembly and equipment for making 1.3-cm. test pellets has been previously described (2). The tests were made using the weak acids, theophylline, theobromine, and phenobarbital. These substances were recrystallized twice from boiling water and dried at 105°. Benzoic acid which was also used was recrystallized twice from boiling water and dried under partial vacuum over sodium hydroxide pellets at room temperature. The melting points of all compounds were in accord with literature values. The tribasic sodium phosphate used was the dodecahydrate of analytical reagent grade. The mixtures of acid with tribasic sodium phosphate were made of −200 mesh particles on a mole fraction basis.

Dissolution Rate Determinations.—Theophylline, theobromine, and benzoic acid-sodium phosphate mixtures were studied by the method previously used for theophylline salts in which solution took place from a constant surface in stirred medium (2). Phenobarbital-sodium phosphate mixture rates were determined by a method in which free convection, diffusion constant, and concentration in the diffusion layer were the rate-determining factors (3). A few additional tests of the first mentioned mixtures were also made by this method. The rates per unit area or fluxes were calculated from the slopes of concentration *versus* time curves and expressed as Gm./sec.-cm.^{2} for tests from stirred medium. Fluxes from determinations by free convection were available directly after correcting apparent rate by multiplying by the product of the ratio of the density of the pellet to the difference between the density of the pellet and medium and weight fraction acid in mixture. All determinations were made in water at 25° unless otherwise noted.

Analytical.—The concentration of acids in dissolution medium were determined spectrophotometrically. Theophylline was determined at 270 mμ (4) and theobromine at 273 mμ (5) after adjusting the solutions to 0.1 *N* with respect to HCl. Benzoic acid was also determined in 0.1 *N* HCl after establishing that the absorbance of its solutions at an absorption maximum at 230 mμ were linear to densities of at least 0.8. Its calculated molar extinction was 11,100 which was lower than a previously reported value of 11,600 (6). The value determined here was probably the more correct of the two, since it was obtained with calorific grade benzoic acid.

Pellet Density Determination.—The density of pellets was determined by measurements of thickness and diameter and weighing. The surface of

* Received August 5, 1957, from the School of Pharmacy, University of California Medical Center, San Francisco 22.

each pellet consisting of acid in the various mixes was calculated by the following expression:

$$F = \frac{\frac{w_a}{d_a}}{\frac{w_a}{d_a} + \frac{w_p}{d_p}} \qquad \text{(Eq. 1)}$$

In Eq. 1, F was the fraction of surface that consisted of acid in a compressed pellet, w_a was the weight of acid in the mixture, w_p the weight of tribasic sodium phosphate in the mixture and d_a and d_p, respectively, the apparent densities of theophylline and tribasic sodium phosphate after compression at the same pressure as the pellet mixes.

RESULTS AND DISCUSSION

Rate Determinations.—The concentration of drug in dissolution medium was found to increase linearly with time for all materials studied by the stirring process indicating that the reduced form of the Noyes-Whitney solution rate equation,

$$\frac{da}{dt} = KSC_s. \qquad \text{(Eq. 2)}$$

described the process from any given two-component system. Equation 2 describes rate of solution taking place from a constant surface into a medium whose concentration is always low with respect to dissolving solid. In Eq. 2, a is the amount of material in solution, t is time, K is an apparent rate constant incorporating among other things the rate constants for both dissolving species, S is surface, and C_s is concentration of the compound of interest in the diffusion layer. The linearity of the relationship between concentration and time is shown in Fig. 1 for several compositions studied.

The fluxes calculated from concentration *versus* time plots for theophylline, theobromine and benzoic acid are plotted as a function of mole fraction of each acid in the mixtures in Fig. 2. Data for phenobarbital are also included. It was not necessary to calculate flux for this compound from concentration *versus* time plots since the experimental determination gave a rate directly convertible to the ordinate term shown as previously discussed.

The curves of Fig. 2 indicate that a complex relationship existed between flux and fraction of drug in a mixture. Further, a marked enhancement in rate was obtained in all cases when compared to the free acid. In Fig. 3 the observed flux for theophylline has been corrected to unit area theophylline and plotted *versus* fraction in mixture. In the region of linearity on this plot the rate-determining factor was the fraction of a pellet's surface that consisted of theophylline. Similar plots for the other materials studied could only be fitted by straight lines in more limited regions. The observed flux for all acids could be expected to be influenced by the hydrogen ion concentration existing in the diffusion layer. It was not practical to measure this quantity.

Examination of pellets after tests in stirred medium did not show surface roughness that would indicate that solution took place by a process other than near simultaneous release of both components. The composition consisting of $^2/_3$ mole phenobarbital with $^1/_3$ mole tribasic sodium phosphate

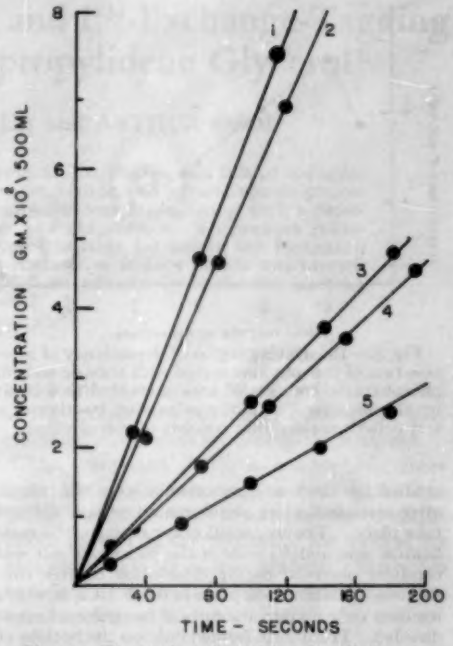

Fig. 1.—Illustrating depression in solution rate when 0.1 N HCl instead of water was used for dissolution medium with aminophylline and a theobromine composition consisting of $^1/_3$ mole theobromine with $^2/_3$ mole tribasic sodium phosphate. Curve 1—aminophylline in water, Curve 2—aminophylline in 0.1 N HCl, Curve 3—theophylline $^2/_3$ mole plus tribasic sodium phosphate $^1/_3$ mole in water, Curve 4—theobromine $^1/_3$ mole plus tribasic sodium phosphate $^2/_3$ mole in water, and Curve 5—the composition in 4 in 0.1 N HCl. Curves 3, 4, and 5 also illustrate the linear release with time of weak acids from these two component systems.

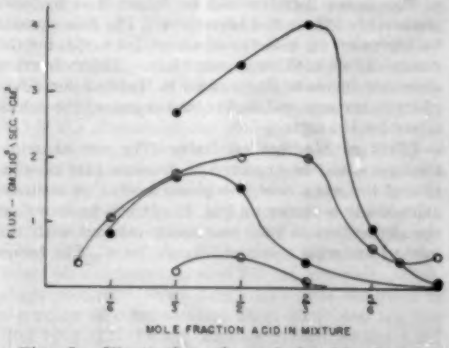

Fig. 2.—Illustrating the relationship between rate and fraction of acid for mixtures of weak acids and tribasic sodium phosphate. Dissolution in water at 25°. Curves from top to bottom are, respectively, for benzoic acid, theophylline, theobromine, and phenobarbital.

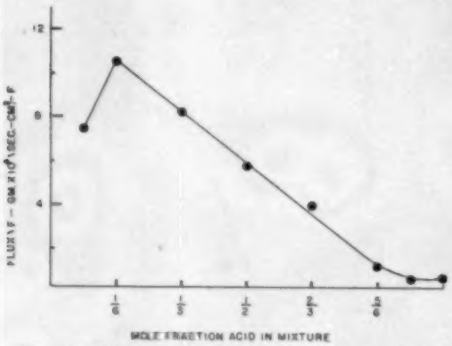

Fig. 3.—Illustrating region of dependency of solution rate of theophylline mixed with tribasic sodium phosphate on fraction of area presented to solvent by theophylline. *F* is the calculated fraction of a test pellet's surface that consists of theophylline.

studied by the free convection process was pitted after tests indicating simultaneous release did not take place. The composition consisting of $^2/_3$ mole benzoic acid and $^1/_3$ mole of the phosphate salt was carefully observed during dissolution by free convection. Solution took place in 0.1 *N* HCl, in which medium only minute amounts of benzoic acid could dissolve. Precipitate formed only on the bottom of the dissolution container with the streaming diffusion layer remaining clear as it fell through medium. This indicated that simultaneous solution of both components took place at the solid-liquid interface.

Comparison Between Fluxes of Weak Acid-Phosphate Mixes and Salts of the Weak Acids.—A comparison of fluxes obtained previously for the dissolution of theophylline salts with the maximum flux obtained with the phosphate mixes shows that even the slowest-dissolving theophylline salt, aminophylline, dissolved much faster than the mixture possessing the most rapid rate. This is shown in Table I where the fluxes for the benzoic acid and phenobarbital sodium salts, as well as the ethylenediamine salt, are compared with the maximum rates per unit area obtained using phosphate mixes.

The fluxes listed would be larger if anhydrous sodium phosphate had been used. The fluxes would be increased by a factor of about 1.4 to 1.8 for the compositions with maximum rates. This correction does not increase flux values in Table I for acid-phosphate compositions to equal or exceed the values found for the salts.

Effect of Medium on Rate.—The use of acidic medium could be expected to decrease rate of solution of the weak acid-phosphate mixes. A comparative study is shown on Fig. 1. It will be seen that the depression in rate was more marked with the mixes than with a salt of theophylline. The buffer capacity of the diffusion layer was no doubt greater in the true salt.

TABLE I.—COMPARISON OF SOLUTION RATE PER UNIT AREA VALUES—TRIBASIC SODIUM PHOSPHATE-ACID MIXES *vs.* SALTS OF ACIDS

Compound or Composition	Rate[a]
Ethylenediamine theophylline (aminophylline)	6.53[b, c]
Theophylline $^2/_3$ mole-tribasic sodium phosphate $^1/_3$ mole	2.03[b, c]
Sodium phenobarbital	4.3[b, d]
Phenobarbital $^1/_3$ mole-tribasic sodium phosphate $^1/_3$ mole	0.48[b, d]
Sodium benzoate	1.85[d, e]
Benzoic acid $^2/_3$ mole-tribasic sodium phosphate $^1/_3$ mole	0.26[d, e]

[a] Gm./sec.-cm.^{2} × 10^4 of acid at 25°.
[b] Dissolution in water.
[c] Dissolution in 0.1 *N* HCl.
[d] Dissolution by free convection.
[e] Dissolution in stirred mediums.

Effect of Acid Strength on Amount of Phosphate Required to Obtain Maximum Flux.—An examination of Fig. 2 will show a decrease in amount of phosphate needed to obtain maximum flux with increase in strength of acids. The literature values for pK's of theobromine, theophylline, and benzoic acid are, respectively, 10.1, 8.77, and 4.19 (7). The acid strength of phenobarbital does not correlate with this grouping, but it was studied by a different process.

SUMMARY

The dissolution rate of weak acids was found to be considerably enhanced by the addition of tribasic sodium phosphate before compression into pellets. The relationship between rate and composition was complex showing a maximum whose location appeared to be related to acid dissociation constant. When the rate of the composition of a given acid-phosphate mixture showing maximum rate was compared to the rate found using the true salt, the latter rate was always greater. This was true when rates were compared from a given constant surface area.

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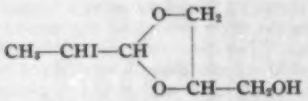
A Study of Certain Physical and I^{131}-Exchange-Tagging Characteristics of Iodopropylidene Glycerol*

By GEORGE F. HOFFNAGLE† and ARTHUR OSOL

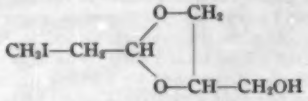
Preparatory to an investigation of the biological distribution and fate of iodopropylidene glycerol in rats, a study of the ultraviolet, visible, and infrared spectrophotometric absorption characteristics of the compound was made, along with a determination of its distribution between water and chloroform. To prepare radioactive iodine-labeled iodopropylidene glycerol suitable for use in the biological study, a process of exchange tagging with radioactive sodium iodide was investigated, and the conditions for obtaining optimum radioactivity in the test material established.

IODOPROPYLIDENE GLYCEROL (hereafter called IPG) has been demonstrated to be a safe and orally efficacious source of iodine for nutritional and therapeutic use in humans (1–4).

The IPG supplied commercially[1] consists principally of 1,2-(2-iodopropylidene)glycerol or 2 - (1 - iodoethyl) - 1,3 - dioxolane - 4 - methanol (2IPG)



with a minor component of 1,2-(3-iodopropylidene) glycerol or 2-(2-iodoethyl)-1,3-dioxolane-4-methanol (3IPG).



No information concerning optical isomers which may be present is available (2IPG has eight possible stereoisomers, 3IPG has four).

The commercial preparation is routinely assayed for IPG content by digestion in a strongly alkaline, aqueous solution at boiling temperature, followed by titration of iodide with silver nitrate V. S. in the presence of nitric acid and a small amount of elemental iodine, with starch T.S. as indicator. The preparations for this study, all of them viscous liquids, contained approximately 34 to 37% of iodine. Since the theoretical content of iodine in the isomeric acetals is 49.2%, the products supplied contained 70 to 75% of the acetals; the remainder consisted of noniodinated compounds incident to the synthesis.

The compound 3IPG is considerably more susceptible to decomposition by alkali than 2IPG; because of this it was possible to obtain 2IPG, as well as the mixture. No method for obtaining 3IPG alone was available. Attempts to separate the mixed isomers by distillation were unsuccessful.

EXPERIMENTAL

Concentration of the Acetals; Distribution between Chloroform and Water.—In order to concentrate the acetals present in the preparations provided for these studies, chloroform solutions of the latter were successively extracted with water to remove nonacetal components. The acetals were recovered from the chloroform solutions by distilling the solvent under vacuum with application of mild heat. This procedure was found to be capable of concentrating the iodinated compounds to as high as 92.5%, as shown by alkaline hydrolysis followed by titration of iodide with silver nitrate V.S. The yield of acetals by this extraction procedure was, however, only about 60% of theoretical. The reason for this loss was found after study of the distribution of a 92.5% material between chloroform and water, the distribution ratio for chloroform/water being observed to be approximately 10.

Ultraviolet and Visible Absorption Characteristics.—A spectrophotometric absorption curve (Fig. 1a) in the ultraviolet and visible regions for an ethanol solution of a preparation containing 71% of mixed IPG isomers was prepared from measurements made with a Beckman DU spectrophotometer. A single absorption peak was found at a wavelength of 255 mμ, and a minimum at 233 mμ. The absorptivity (1%, 1 cm.) at 255 mμ was found to be 19.1 based on a concentration of 366 mg. per liter. Only slight deviation from Beer's Law was observed in the range of concentration from 50 to 450 mg. per liter (Fig. 1b). Other samples agreed within 1.2% based on their IPG content.

Infrared Absorption Characteristics.—Infrared spectrophotometric absorption curves (Fig. 2) were obtained for three samples of the preparation as noted using a Baird instrument. The samples were

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Presented to the Scientific Section, A. PH. A., New York meeting, May 1957.
Abstracted in part from a dissertation submitted by George F. Hoffnagle to the Graduate School of the Philadelphia College of Pharmacy and Science in partial fulfillment of the requirements for the degree of Doctor of Science.
This investigation was supported in part by a grant from Henry K. Wampole & Co., Inc., Philadelphia, Pa.
† Present address: 86 Forest Ave., Verona, N. J.
[1] Preparations of IPG used in this study were supplied by Henry K. Wampole & Co., Inc., Philadelphia, Pa.

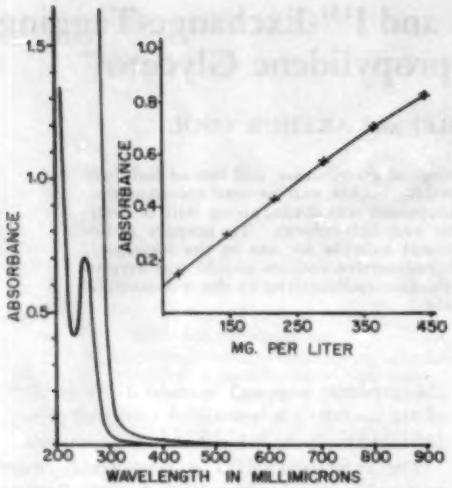

Fig. 1a.—Absorption characteristics of IPG at concentrations of 366 mg. per liter and 3660 mg. per liter in ethanol in the ultraviolet and visible regions.

Fig. 1b.—Variation of absorbance with concentration of IPG in ethanol at a wavelength of 255 mμ.

placed undiluted between sodium chloride plates. By means of these curves, it appears to be possible to distinguish between IPG and compounds incident to the synthesis, and to accept the top curve as a good approximation of the absorption characteristics of IPG. The tentatively identifiable peaks are noted in Fig. 2. The carbonyl peaks can be removed either by mild alkaline treatment or by solvent extraction, and can be estimated by the hydroxylamine reaction; the carbonyl is not in the IPG structure. The hydroxyl peak can be reduced but not removed by concentration procedures, indicating its presence both within and outside the IPG structure. Absorption by the iodine atom is beyond the range of the instrument, but residual chloroform from the concentration procedure is present in the top and center curves. The peaks at 6.19 μ and in the range of 8 to 10 μ are common to cyclic ethers such as dioxanes and aliphatic ethers.

Investigation of Tagging of IPG with Iodine-131.—The ultimate purpose of this phase of the study was to provide a radioactive material of sufficient activity to trace IPG in biological experiments.

Exchange tagging of 2IPG was studied under four conditions. In formulation 1 the IPG and elemental I^{131} were mixed in a mildly alkaline medium; in formulation 2 the IPG and I^{131} as iodide were mixed in a mildly alkaline medium; in formulation 3 the IPG and elemental I^{131} were mixed in a mildly acid medium; in formulation 4 the IPG and I^{131} as iodide were mixed in a mildly acid medium. The compositions of the media used are set forth in Table I.

The solutions were allowed to stand in glass-stoppered bottles at room temperature. To determine the extent of exchange-tagging the following procedure was used. Samples of 20 ml. each were removed from each solution at four, seven, and ten days. The solutions were treated to remove inorganic iodine as follows. Dilution of the inorganic iodine was accomplished by the addition of 100 mg. of potassium iodide. Since the amount of I^{131} at the start of the experiment was approximately 5.4×10^{-7} mg., this dilution amounts to some 1.4×10^{8} times. The solution was then made acid with acetic acid, 0.5 ml. of starch T. S. added, and sufficient potassium permanganate T. S., to obtain a blue color. A slurry of silver carbonate was then added dropwise, slowly and with agitation, until the blue color just disappeared. The mixture was diluted to 25 ml. with distilled water and quickly filtered through a Seitz filter, using a K5 pad, thereby removing the silver iodide and any unreacted silver carbonate. This procedure, when followed meticulously, yielded a solution which would neither darken on exposure to light, nor show any precipitate on adding a small amount of sodium chloride. Also, the radioactivity could be reduced to approximately background activity by extracting the solution with four 10-ml. portions of chloroform, thereby indicating that no appreciable portion of the radioactivity was present as iodide. Furthermore, paper chromatograms (5) showed no detectable iodide, iodate, or free iodine. The total iodine content in the final solutions was determined by alkaline digestion followed by titration with 0.01 *N* silver nitrate. The K5 pads were found to have absorbed only an estimated 0 to 5% of IPG from the solutions.

The radioactivity of the sample, diluted with 0.1% sodium iodide solution, was determined using an organic-quenched, end-window, Geiger-Mueller counter tube, the sample being 2 ml. of solution held in a plastic planchet. An equilibrium sample of RaDEF was used as the standard for calibrating the equipment, and the samples were converted to curies of activity, where desired, by comparing with dilutions of the carrier-free sodium iodide-^{131} supplied by Abbott Laboratories, using the value of the standard

TABLE I.—COMPOSITION OF MEDIA EMPLOYED IN TAGGING IPG WITH I^{131}

Ingredients →	Composition of Formulation 1	2	3	4
2IPG	0.15 Gm.	0.15 Gm.	0.15 Gm.	0.15 Gm.
Iodine equivalent	0.0738 Gm.	0.0738 Gm.	0.0738 Gm.	0.0738 Gm.
Glycerin	45 ml.	45 ml.	45 ml.	45 ml.
Distilled water	15 ml.	15 ml.	15 ml.	15 ml.
Sodium bicarbonate	1 Gm.	1 Gm.	.....	.....
Glacial acetic acid	.....	.....	1 ml.	1 ml.
Carrier-free NaI^{131}	0.2 mc.	0.2 mc.	0.2 mc.	0.2 mc.
Potassium permang. T. S.	0.2 ml.	.....	0.2 ml.	.....

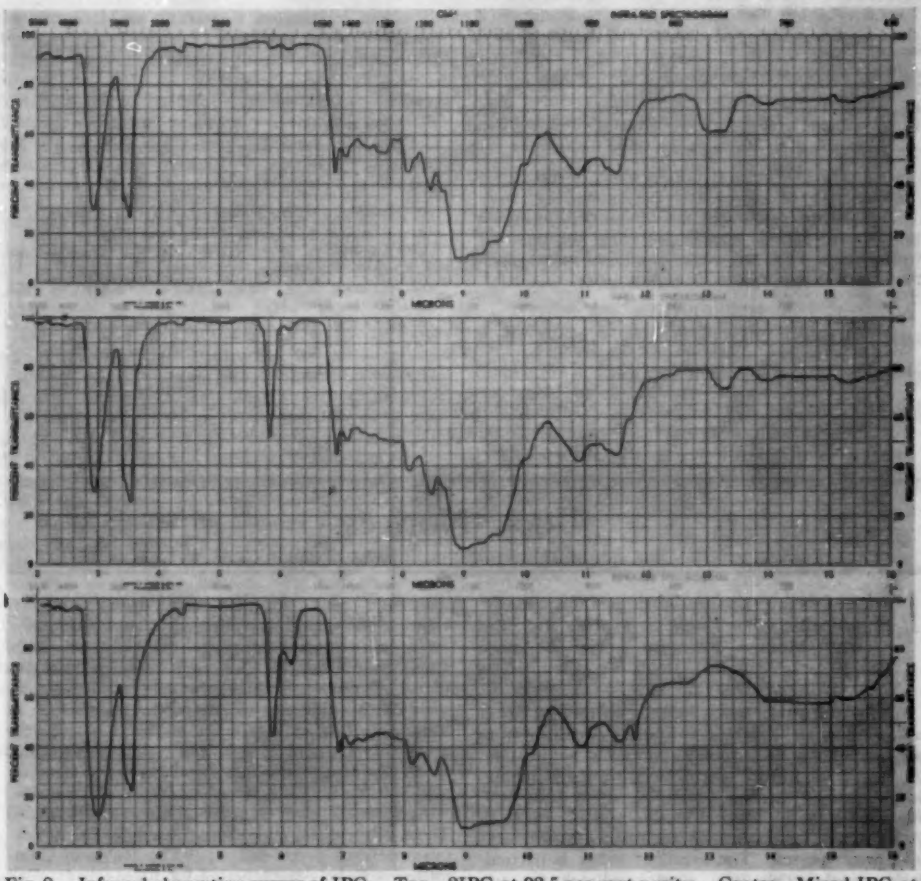

Fig. 2.—Infrared absorption curves of IPG. Top—2IPG at 92.5 per cent purity. Center—Mixed IPG at 85.8 per cent purity. Bottom—Mixed IPG at 71.2 per cent purity.

supplied by them, corrected for decay. The results of the tagging are in Table II.

The same experiments using mixed IPG yielded the same results in acid media, but even lower results in alkaline media because of the lability of 3IPG under these conditions.

TABLE II.—EXTENT OF TAGGING OF IPG WITH I^{131} IN VARIOUS MEDIA, IN MICROCURIES PER MG. OF IODINE IN IPG, NOT CORRECTED FOR DECAY

Exchange Time	Formulation 1	2	3	4
4 days	0.264	0.168	0.364	0.291
7 days	0.072	0.143	0.350	0.304

A second type of experiment was undertaken employing the optimal conditions for tagging as represented by formulation #3 above, but adding varying amounts of I^{131} to a constant amount of 2IPG (60 mg.); the amounts used and the results, using the same sampling methods as above, are found in Table III.

TABLE III.—EXTENT OF TAGGING OF IPG USING DIFFERENT INITIAL AMOUNTS OF I^{131}, IN MICROCURIES PER MG. OF IODINE IN IPG, NOT CORRECTED FOR DECAY

Sample	Radioactivity added initially to 60 mg. 2IPG	Exchange Time — 36 Hr.	72 Hr.	120 Hr.
I	0.4 millicurie	0.69	0.86	0.87
II	2.0 millicuries	2.0	2.5	2.5
III	4.0 millicuries	3.0	3.8	4.3

These data clearly pointed to the probability of obtaining even higher specific activities of tagged IPG by increasing the proportion of radioactive sodium iodide to IPG. Further experimentation proved this to occur. However, this exchange reaction could not be characterized as zero-order, first-order, or second-order.

This information was used to produce tagged IPG of high specific activities for chromatographic (5) and biological (6) use. The results of a series of such experiments with varying proportions of NaI^{131} to IPG and varying times are listed in Table IV.

TABLE IV.—I^{131} TAGGING OF 2IPG TO PRODUCE HIGH SPECIFIC ACTIVITIES, REPORTED AS MICROCURIES PER MG. OF IODINE IN IPG, NOT CORRECTED FOR DECAY

Added radioactivity	9 mc.	9 mc.	8.3 mc.	9 mc.	6.9 mc.
Iodine in 2IPG	53.7 mg.	31.6 mg.	34.8 mg.	54.0 mg.	39.8 mg.
Exchange Time	7 days	8 days	12 days	13 days	16 days
Final radioactivity	15.4 uc.	23.8 uc.	24.4 uc.	8.0 uc.	7.0 uc.

The raw material ratio and exchange time which produce the highest specific activities are apparent from Table IV, and were employed when such material was required.

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Book Notices

Medical Writing, The Technique and the Art. 3rd ed. By MORRIS FISHBEIN. The Blakiston Division, McGraw-Hill Book Co., Inc., New York, 1957. x + 262 pp. 15 x 23 cm. Price $7.

The sound advice given by the experienced editor and author, Dr. Fishbein, can apply equally well to anyone who is preparing a report for publication. Although the author's experience has been mainly in the field of medical journalism, the terminology, illustrations, etc., utilized in this text are medical. A brief chapter deals with pharmaceutic products and prescriptions. The excellent index is particularly useful. This is a good handbook for any editorial office.

The National Formulary of Japan 2nd ed. Compiled and edited by the Formulary Section of National Council of Pharmacy. English edition published by the Ministry of Health and Welfare, Tokyo, 1957. 14.5 x 21 cm. vii + 167 pp.

The second edition of the N. F. (Japan) is evidence of the progress that has been made in the introduction of new medicinals into the Japanese materia medica, although some folklore medicinals are still noted; e.g. Japanese Ginseng and Oyster Shell. The text monographs are arranged according to the Latin title of the drug or dosage form, and this makes one realize the greater convenience of the arrangement in the N. F. (U. S.). The English title is not always a translation of the Latin; e.g. *Tiglium*, Croton Seed; with the synonym Tiglii Semen. *Epirenaminae bitartras* has the English title Epirenamine Bitartrate, but *Collunarium epirenaminae compositum* has the English title Compound Epinephrine Nose Drops. Epirenamine is not included among the 36 names for epinephrine that are given in the Merck Index 6th ed.

The monographs represent excellent technical study and preparation, but it is surprising to note that the formulas and equivalence factors are based on atomic weights adopted by the International Committee in 1949; although the investigative phase of revision was continued into 1954. In view of the difficulty experienced with the use of sodium acetate as the buffer in the N. F. (U. S.) assay for methionine, it is interesting to note that the N. F. (Japan) monograph utilizes the phosphate-acid phosphate buffer, which probably is preferable.

Appendixes give General Notices and General Tests, Processes and Apparatus of The Pharmacopoeia of Japan 6th ed. The Revision Committee is to be congratulated for its excellent work in preparing this edition of the N. F. (Japan).

The Principles of Therapeutics. By J. HAROLD BURN. Charles C Thomas, Publisher, Springfield, Ill., 1957. ix + 278 pp. 14 x 22.5 cm. Price $5.50.

This book is a compilation of the author's lectures on "Pharmacology and the Basis of Therapeutics." The subjects included are: Development of therapeutics; Ion exchange in cellular activity, active substances in the body; Transmission of nerve impulses by acetylcholine and noradrenaline; Properties of acetylcholine and of substances modifying its action; Treatment of hypertension; Substances whose action resembles that of acetylcholine, especially nicotine; Adrenaline and the sympathomimetic amines; Action of histamine, anaphylaxis, allergy, histamine liberators and antihistamine substances; Hydroxytryptamine and bradykinin; Pituitary (posterior lobe) extract, Biological standardization; Digitalis glycosides; Substances relaxing smooth muscle including vasodilators; Analgesic substances; Cocaine and the local anesthetics; Alcohol; Anesthetics; Barbiturates and tranquillizing drugs; Stimulant drugs; Drugs of the alimentary canal; Action of drugs on

the uterus, ergot; The treatment of anemias; Thyroid Gland; Insulin; Oestrogens and rogens; Adrenal cortex; Disinfectants; Sulfonamides and salicylates; Kidney, treatment of oedema; Antibiotics; Malaria; Chemotherapy in tropical diseases; Metallic salts and vitamins; Biological variation and clinical trials. The book is intended for medical students and physicians. It should be a useful addition to the Pharmacy library as well.

Quantitative Organic Analysis. By JAMES S. FRITZ and GEORGE S. HAMMOND. John Wiley & Sons, Inc., New York, 1957. ix + 303 pp. 14.5 x 23 cm. Price $6.50.

This textbook utilizes the principle of analysis of organic compounds by selective, functional group reactions. The following chapter headings indicate the scope of the text: Details of a method (analytical), Acid-base titrations in nonaqueous solvents, Indirect acid-base methods, Oxidative and reductive methods, Manometirc methods, Metal ion complexes, Elemental analysis, Kinetics in analysis, Spectrophotometric methods, Separations, Physical and polarographic methods, Solving new analytical problems, and Laboratory procedures. The text is designed for use at the later undergraduate and graduate level of instruction. It is documented throughout with references to the literature. Problems are included at the end of each chapter. Author and subject indexes are appended. The book should be considered by teachers of organic analysis and newer analytical techniques.

Recent Progress in Hormone Research, Vol. XIII: Proceedings of the Laurentian Hormone Conference 1956. Edited by GREGORY PINCUS. Academic Press Inc., New York, 1957. v + 646 pp. 15 x 23 cm. Price $12.80.

The papers and discussions of the thirteenth annual Laurentian Hormone Conference comprise this volume. The twelfth volume was reviewed in THIS JOURNAL, *46*, 149(1957). The reports, grouped under five main headings, are: I. Neurohumoral-Endocrine Relationships.—Biochemical, physiological, and pharmacological aspects of serotonin; Effects of midbrain and spinal cord transection on endocrine and metabolic functions with postulation of a midbrain hypothalamico-pituitary activating system; Studies on the influence of the central nervous system on anterior pituitary function; Hormones and rhythms in man and animals. II. Hormone Transport in Circulation.—Interaction of thyroid hormones and protein in biological fluids; Binding of steroids and steroid conjugates to human plasma proteins. III. Aspects of Reproduction.—Some experimental studies on the Mechanism of ova-implantation in the rat; Synthetic progestins in the normal human menstrual cycle; Metabolism of progesterone and its clinical use in pregnancy; Long-acting steroids in reproduction. IV. Hormone Chemistry and Metabolism.—Insulinase, insulinase-inhibitors, and diabetes mellitus; Glucagon, a second pancreatic hormone. V. Hormones and Stress.—Endocrine changes after anesthesia, surgery, and unanesthetized trauma in man; Adrenal influences upon the stomach and the gastric responses to stress.

The excellent style, format, and binding of this series is continued. The references at the end of each paper and the appended author and subject indexes add to the value of this volume, and the other volumes in this series, as additions to the reference library in this field.

The Lipids. Their Chemistry and Biochemistry. Vol. 3, Biochemistry (Biosynthesis, Oxidation, Metabolism, and Nutritional Value). By HARRY J. DEUEL, JR. Interscience Publishers, Inc., New York, 1957. xxxvi + 1065 pp. 15.5 x 23.5 cm. Price $25.

This is the third volume of a comprehensive text on the lipids, and completes the coverage of the biochemical phase of the subject. The author, Dr. Deuel, died in 1956, and some of the later material was added by an editorial committee. The fine style, detailed scope, and excellent printing and format of this volume are similar to that in vols. 1 and 2 which were reviewed, respectively, in THIS JOURNAL, **41**, 57(1952) and **44**, 318(1955). Of particular interest at this time is the final chapter on the nutritional value of fats. The many references throughout the text and the extensive author and subject indexes make this set of books indispensable as a reference work.

Experimental Control of Plant Growth. By FRITS W. WENT. Chronica Botanica Co., Waltham, Mass., 1957. xvii + 343 pp. 15 x 23 cm. Price $8.50.

This book presents in detail the factors affecting plant growth and describes the methods used in the experimental studies at the Clark Greenhouses and the Earhart Plant Research Laboratory. In addition to author and subject indexes, 27 pages of photographs are appended.

Practical Clinical Chemistry: A Guide for Technicians, 2nd ed. By ALMA HILLER. Charles C Thomas, Springfield, 1957. xvii + 265 pp. 16 x 23.5 cm. Price $6.50.

The various tests and procedures utilized in clinical chemistry are described in detail and outlines for the different determinations are included. Duplicate forms of the outlines are appended for removal from the book. A general subject index is included. The book should be a useful textbook and laboratory manual as well as a guide for practicing clinical technicians.

Cortisone Therapy: Mainly Applied to the Rheumatic Diseases. By J. H. GLYN. Philosophical Library, Inc., New York, 1957. x + 162 pp. 14 x 22 cm. Price $10.

The relative therapeutic value of cortisone, corticotrophin, hydrocortisone, prednisone, and prednisolone, primarily in the treatment of rheumatic diseases are reviewed, with detailed regimens of treatment included. It is an important contribution to the rational consideration of a complicated problem in medical practice by a practitioner who is personally familiar with the practical aspects of this field of therapy. A general index is appended.

Actualités Pharmacologiques. Series No. 10. RENÉ HAZARD, Editor. Masson et Cie, Paris-6, France, 1957. 16.5 x 25.4 cm. 269 pp.

This tenth volume of the series (in French) contains contributed chapters on: Mode of action of substances protecting living bodies against ionizing radiations, Chemical mediators and central nervous activities in vertebrates, Organophosphorus anticholinesterases, Pharmacologic action of spinally administered medicaments, Metabolic transformations of organic toxic substances (general biology and industrial hygiene), Pharmacodynamics and comparative physiology, Sulfamide inhibitors of tubulary functions, inhibitors of carbonic anhydrase, and hypoglycemic agents, Human plasma fractions and their therapeutic uses. The paper and printing are good, but the volume has a paper back. An alphabetical index of authors of chapters in vols. 1–10 is appended.

The Relation of Psychiatry to Pharmacology. By ABRAHAM WIKLER. The Williams & Wilkins Co., Baltimore, 1957. viii + 322 pp., Paper back. 17 x 25 cm. Price $4.

This book, the printing of which was subsidized by the American Society for Pharmacology and Experimental Therapeutics, is a comprehensive review of the relation of psychiatry to pharmacology with special considerations of the drugs used in this field of medicine. The types of drugs include tranquilizers, mood elevators, anesthetics, psychosomimetic agents, analeptic agents, and insulin. The text is divided into two main sections headed: The effects of drugs on human behavior, and Theories and mechanisms of drug actions. It is ended with a 4-page chapter on General conclusions. The 889 references in the text are given at the end, and a 12-page index is appended.

Biochemistry for Medical Students. 6th ed. By W. V. THORPE. J. B. Lippincott Company, Philadelphia, 1956. viii + 542 pp. 14.5 x 21 cm. Price $6.75.

The book represents a revision of a standard textbook on biochemistry. The major additions to the 6th edition relates to protein structure, enzyme A and its role in metabolic processes. The entire chapter on enzymes has been thoroughly revised and contains much essential new information on this subject. In addition, the material on nucleic acids has been rewritten and the subject of redox potentials has been brought up to date. The bibliography is adequate for the purpose intended and includes references to recently published works.

Klink und Therapie der Vergiftungen. By SVEN MOESCHLIN. Georg Thieme Verlag, Stuttgart, 1956. Intercontinental Medical Book Corporation, New York. xi + 521 pp. 18.5 x 25 cm. Price DM 59. Price $14.05.

This book is designed for professional toxicologists, physicians, and others concerned with hazards arising in the use of household chemicals and drugs and those met with by workers in the chemical and allied industries. The content of the book is adequate for its intended purpose and the subject matter appears to be arranged in a logical order. It begins with discussions of the frequency and distribution of poisoning, general principles employed in the treatment of poisoning, and a listing of the more important materials and preparations used in the treatment of cases of poisoning. These general chapters are followed by a useful table of maximum allowable concentrations, expressed in parts per million, of gases, vapors, and dusts in the air to which workers may be exposed without creating a health hazard through inhalation of the potential poisons listed. Proceeding from this table the book is divided into sections, each with several subdivisions, relating to inorganic chemicals, organic chemicals, vitamins (AB&D), hormones, poisons from plant sources and the derivatives, antibiotics, food poisons, and animal poisons. Sources of exposure are then discussed.

The book is thoroughly documented throughout, and is provided with an author index and an unusually complete subject index. The paper, printing, and binding are of excellent quality representative of all books produced by the Georg Thieme Verlag. The author, Dr. Sven Moeschlin, of the University of Zurich, is to be commended for having done a thorough job of treating an important subject. The book can be recommended to those who are concerned with the diagnosis and treatment of cases of poisoning.

Antiseptics, Disinfectants, Fungicides, and Chemical and Physical Sterilization. 2nd ed. Edited by GEORGE F. REDDISH. Lea & Febiger, Philadelphia, 1957. 975 pp. 15 x 23.5 cm. Price $15.

This edition of an excellent book has been brought up to date by twenty-eight contributors. It includes many recent advances in areas treated earlier. Three new chapters cover: Sterility tests and methods of assuring sterility, Methods of testing sanitizers and bacteriostatic substances, and Ultraviolet radiation as a germicidal agent. The fine style, type, printing, format, and binding are similar to that of the first edition, which was reviewed in THIS JOURNAL, **43,** 445(1954). The chapter on iodine includes an extensive discussion of iodophors and the use of iodine solutions during catastrophies. A new section under phenolic compounds deals with the official evaluation of phenolic disinfectants. A useful tabulation in the section on quaternary ammonium compounds lists substances that are compatible and those that are incompatible with benzalkonium chloride. The many references after each section in the text and the appended index enhance the usefulness of this authoritative book.

Fads and Fallacies in the Name of Science. By MARTIN GARDNER. Dover Publications, Inc., New York, 1957. x + 363 pp. 14 x 20.5 cm. Price $1.50.

The publisher, in some small measure, believes that this debunking publication can counterbalance the endless amount of pseudoscientific drivel and that this small voice of reason will be heard in the cacophony of crackpot ideas. The first edition of this book was published in 1952. This edition contains a new chapter on the Bridey Murphy mania.

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